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PATHOLOGY

A Periodical Devoted to General and Experimental Pathology

SHIELDS WARREN FESTSCHRIFT

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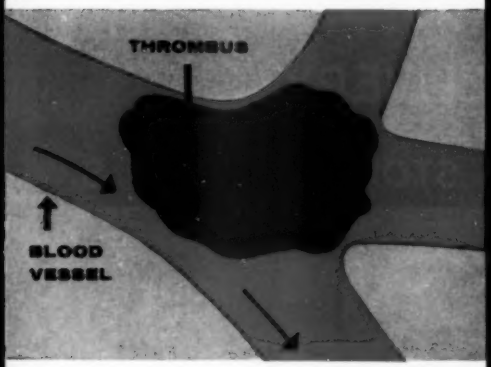

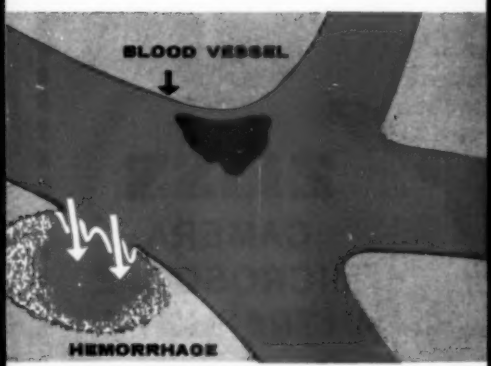

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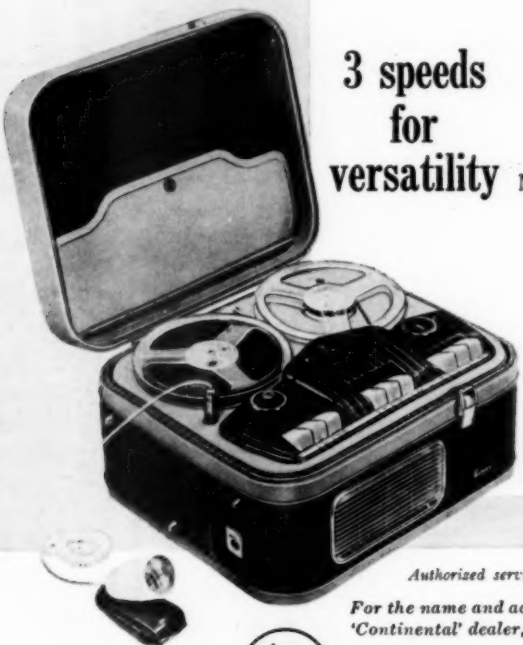

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SHIELDS WARREN, M.D.

A.M.A. ARCHIVES OF
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Foreword

Shields Warren, M.D.

The dedication of this issue of the A. M. A. Archives of Pathology in commemoration of the sixtieth birthday of a physician and scientist who will stand as one of the great figures of pathology derives from the devotion of his graduate pupils, a group of outstanding pathologists who comprise the *Shields Warren Club*. The honor they have conferred upon a colleague of more than thirty years, to whom they have entrusted this introduction, gives me the opportunity to acknowledge that, in common with colleagues throughout the world who did not have the privilege of training directly under him, I, too, am proud to claim Shields Warren as a teacher. For so genuine has been his interest in the progress of medicine everywhere, and so generous has he been in the sharing of his wisdom and experience, that large numbers of pathologists and scientists join with medical students and the fortunates of the "Club" who received their professional training under him in grateful acknowledgement to Dr. Warren, the teacher. All of these were joined by over seven hundred representatives of the worlds of education; medicine; science; the armed forces; the federal, state, and city governmental bodies; the Atomic Energy Commission; the National Institutes of Health, and the community as a whole in a gigantic sixtieth birthday party, unique in the history of Boston. What manner of man evoked such a response from his community and his country and such warmth of regard from all over the world?

Submitted for publication Aug. 28, 1958.

Shields Warren is the first in a distinguished family to achieve leadership in medicine and science. His father was a professor of philosophy and dean of the College of Liberal Arts of Boston University, a school founded by Shields Warren's grandfather and now the largest institution of higher learning in New England. It is only fitting, therefore, but still a source of astonishment to those who learn it for the first time, that in addition to his enormous and varied responsibilities, Shields Warren is chairman of the board of trustees of Boston University. His deep interest in the elevation of standards of education there was appropriately recognized in the creation, by his friends and admirers on his sixtieth birthday, of what is hoped will be a continuing Shields Warren Fund at Boston University for the support of distinguished professorship bearing his name.

Shields Warren grew up in New England, and, until World War II, centered his education, training, and profession in and around Boston. He demonstrated, early in his career, two sets of qualities which have characterized his entire personal and professional life. These were concerned first with the attainment of what might be called inapparent leadership and, further, the choice always of creating something new—of pioneering, rather than seeking the comfortable post already fully developed and circumscribed. He had demonstrated an ex-

traordinary ability to merge himself with his surroundings, provided these meet his always rigid standards, and so in attire, speech, and deportment, he at first does not stand out as a man apart from his fellows. He neither seeks attention nor exhibits undeserved interest in a member of a group. A first glance usually shows a quiet, attentive, unobtrusive, somewhat Lincolnesque person, possessed of a shyness which is accentuated at times by a slight hesitation in speech. This disappears imperceptibly as he becomes more accustomed to his surroundings. Without abrupt change in manner after this preliminary impression, an increasing warmth of human qualities, exuding a depth of integrity which communicates quickly an aura of confidence, draws strangers to him. Humility, absence of pretense, and objectivity in all matters except one characterize his behavior. His love of story-telling at appropriate moments is well known, and he is particularly expert in relating droll tales of the Cape Cod native. He is unable, however, to recognize that whenever he repeats a delightful tale heard elsewhere, no matter what the original dialect employed, the story comes out pure Cape Cod.

The pioneering instincts of Shields Warren characterize all of his endeavors. His early life is replete with examples of achievement which make his subsequent career no surprise. This shy self-effacing person was always a leader and in a number of apparently unrelated activities. This leadership has always been characterized by personal behavior and treatment of his fellows which, at the same time, disguised and emphasized his position of eminence in his many undertakings. From early life he displayed the astonishing knack of remaining in the background while exerting his valuable influence, only to be drawn into the limelight by others in moments of crisis or in the natural development of a project.

Characteristic throughout his life has been the generous recognition, both publicly and in more personal ways, of the contributions of his companions and assistants. His successful campaign to provide a football field for Brookline High School when he was a student there is still celebrated as an example of vision, courage, business acumen, and even audacity and farsighted planning for the common good. A multitude of examples might be gleaned from his days as a student at Boston University, from which he graduated in 1918, or from the four years at Harvard Medical School, preceding the award of his medical degree, in 1923. The year between the end of college and the beginning of medical school was a wonderful *Wanderjahr*, when this son and grandson of university educators and Methodist ministers saw his own country, in part as an itinerant man-of-all-work and in part as a hobo. This story should one day be told in its entirety.

It was no accident that Shields Warren chose pathology as his field of professional activity or that he went to the Boston City Hospital to learn the rudiments of his profession under the critical eye of that master of pathology, Frank Burr Mallory. Two years was then the accepted period of training under Dr. Mallory, who was fond of pointing out that if a man could not learn all that Mallory could teach in two years' time, he would never be much of a pathologist.

Dr. Warren began his teaching association with Dr. S. Burt Wolbach, at the Harvard Medical School, who soon adopted Warren as a dear and trusted friend. Dr. Warren was made one of the four full professors of pathology who succeeded Dr. Wolbach in 1948.

But, at the onset of his professional career, Dr. Warren was not interested in first finding a nest planned, built, and maintained by someone else. Here again his extraordinary ability to create something that would fill a great need and open up new vistas of pathology, medicine, and science was apparent. He organized the first full-time department of pathology at the New England Deaconess Hospital, an institution connected with Harvard Medical School but

not one of its main teaching hospitals. To most of his colleagues this seemed the end of the road for a promising young pathologist. The quarters for pathology were unattractive and inadequate. There were no facilities for research, but what Dr. Warren saw was a surgical Staff composed of men such as Dr. Frank H. Lahey and Dr. Daniel Fiske Jones and a medical staff headed by Dr. Elliott P. Joslin. He quickly rendered a quality of service in clinical pathology and surgical and postmortem pathology which was to be copied throughout this and many other countries. Having carried out his first duty for the welfare of the patient by furnishing surgeons and physicians with rapid, accurate, authoritative, and objective clinical and morbid anatomic pathology routine, he was able to institute a series of studies, at first by himself and later with a magnificent succession of eager young pathologists who came to him for professional training.

Four areas of pathology attracted his attention because of the nature of the clinical problems, and these Dr. Warren systematically explored in studies carried on to the present day. The vast number of patients with surgically treated thyroid disease formed the basis of a series of careful clinicopathological correlations. These clarified many of the hopelessly confused classifications then in vogue and gave to the surgeon and to the internist a sound basis for choice of therapy. People with tumors in great number came to him for diagnosis, and there developed soon a masterful proficiency in the art of frozen-section diagnosis, followed by comparison with permanent sections and constant follow-up studies of patients from whom the tumors had been removed. During this early period too, the beginnings of Dr. Warren's interest in the effects of radiation upon the tissues and organs of the body were made. Finally, his association with Dr. Elliott P. Joslin provided a unique opportunity to study diabetes as a whole. From this interest there emerged what has become a classic—"The Pathology of Diabetes Mellitus"—which was first published in 1930. The fourth edition of this book is now in preparation, in collaboration with Dr. Philip M. Le Compte.

Dr. Warren saw the remarkable opportunity for a young pathologist to make extensive studies on the effects of radiation on cancer and on the tissues of the body. When radioactive phosphorus was made available, he used it in the treatment of leukemia and with characteristic vision prepared at once for the new era opened by the use of radioactive isotopes in medicine and research. His monograph concerning the changes in normal tissues induced by radiation, written in collaboration with Olive Gates, Charles Dunlap, and Nathan Friedman, in 1942-1943, is still a standard reference work.

The long years of interest in the biologic effects of radiation prepared Dr. Warren for the great responsibilities he was soon called upon to assume when the atomic bombs were dropped in Japan. He was asked first to observe the survivors in Japan, after his years of duty in the medical corps of the United States Navy, and then to become concerned with the Bikini tests. His entire professional activity and his native talents combined to make him the natural choice for the first directorship of the Division of Biology and Medicine of the U. S. Atomic Energy Commission. Although most of his accomplishments in this area have not yet been made public, a clear glimpse of the importance of his achievements can be obtained through the eyes of his colleagues and the remarks of such men as Adm. Lewis L. Strauss, until recently Chairman of the Atomic Energy Commission.

Characteristically, the planning of the structure and functions of the Division of Biology and Medicine of the Atomic Energy Commission took on a scope which multiplied many times the effectiveness of this major effort in research in this country. Under the inspiration of Dr. Warren,

scientists and physicians were given support of a magnitude demanded by the enormity of the task they were asked to assume, and world leadership in this new branch of research was soon evident.

His deep interest in education and in the practical aspects of medical research in pathology shaped the structure of research institutions throughout the country, so that medical schools and universities were strengthened rather than debilitated by the new activities of the Atomic Energy Commission. Such an achievement was all the more remarkable because of the understandable secrecy which was imposed, for reasons of security, upon much of the research in this area.

For five years, Dr. Warren continued as director of the division and since then has maintained close relations with the Atomic Energy Commission, as consultant. His enormous contributions in this field have been recognized by the United States Government in his selection as the United States representative to the United Nations Scientific Committee on the Effects of Atomic Radiation. It is in this activity that the public has had an opportunity to see Shields Warren, the medical statesman. His integrity and scientific stature have influenced those who are less well informed to adopt his belief that we should not stop atomic tests but should rather regard nuclear energy as a force with which the world must reckon, it is hoped, only in peaceful applications. To do this, he has pointed out, we must learn how to control hazards such as those which may prove to be associated with some radioactive isotopes, such as strontium 90.

When the full story of the medical, scientific, and educational contributions through the Atomic Energy Commission can be told, pathology will indeed be proud of the contributions of this man for the good of this country and the world.

Dr. Warren's studies in the field of cancer began with systematic attempts to make clinicopathological correlations for the guidance of the surgeon seeking the best form of therapy. From studies leading to the clarification of nomenclature, achievement of more precise criteria for prognosis, and observations which led to development of better operative procedures, a far more clear picture of the life history and biological behavior of many forms of cancer followed. To raise the standards of cancer surgery throughout Massachusetts, Dr. Warren, later assisted so ably by Dr. Olive Gates, developed a tumor diagnostic service for the Commonwealth of Massachusetts and became a great force in the development of better care for the patient with cancer in the state institutions. His colossal energy, which has become legendary, is beautifully illustrated by the testimony of the young men in training who, as part of their service, were stationed in the Pondville State Cancer Hospital, some twenty miles from Boston. The resident on duty there would meet regularly with him for a study of the accumulated surgical pathological problems when Dr. Warren had finished his day's work in Boston, after eleven o'clock at night. With unflagging energy and no sign of weariness, Dr. Warren would study the problems placed before him, without hurry, as if this were the only task facing him.

A long experimental program concerning the nature of cancer and the effects of radiation on various types of tumors led, finally, to the inauguration, on June 5, 1951, of the Cancer Research Institute of the New England Deaconess Hospital. The important and beautifully organized laboratories of pathology of the hospital had been, for some time, the immediate responsibility of Dr. Warren's highly respected colleague, Dr. William A. Meissner. The newly created Cancer Research Institute, although closely connected with the Department of Pathology, offered magnificent opportunities for the work of an enlarged staff concerned with the biology of radiation and of cancer in the broadest possible terms, with the aid of techniques derived from the disciplines

of biology, physics, and chemistry, all directed toward this program of experimental pathology.

Dr. Warren's interest in pathology has ranged from the teaching of medical students, the training of men for the American Board of Pathology, postgraduate education for general practitioners and specialists, the practice of pathology, experimental pathology, and participation in the activities of time-honored associations to which teachers and practitioners of pathology belong to helping to found new organizations designed to meet the needs of pathology. And so we find Dr. Warren serving as president of the American Society for Experimental Pathology, the American Association of Pathologists and Bacteriologists, the American Association for Cancer Research, and the American Board of Pathology and identified with the American Society for Clinical Pathology as recipient of its highest recognition, the Burdick Award.

While emphasizing the importance of detailed study of specialized areas of pathology, he has viewed it always as a whole and has followed his interests to special areas, wherever they are. With his associate, Dr. Samuel P. Hicks, he wrote a book on neuropathology from the viewpoint of the general pathologist. The important discovery of Papanicolaou was put to practical test without delay, and from his studies with his colleague, Dr. Olive Gates, there soon emerged an authoritative handbook for the diagnosis of cancer of the uterus.

Dr. Warren has given years of service as a consultant to the United States Army, Navy, Air Force, Veterans' Administration, and National Institutes of Health. He has served as a member of the National Advisory Cancer Council and the National Research Council.

A life so filled with dedication to his profession and to the commonwealth would seem out of balance if it were not for hours, both planned and stolen, spent with Alice, his wife, and his two daughters and their families, at Waquoit, near Woods Hole on the Cape. There Shields Warren becomes the sailor; the fisherman; the host to family, friends, and pupils, and the center of a devoted and happy family group.

We leave him now, apparently at the height of his vigor, physically and mentally, engaged in the daily practice of pathology and the teaching of students and doctors in his chosen field and directing the active investigations of a group of devoted colleagues in a Cancer Research Institute. We see him breaking away for a characteristically brief, efficiently organized and effective consultation with governmental bodies or medical or scientific committees, or for his more prolonged sessions at intervals as a representative of his country in the scientific sessions of the United Nations. We see him presiding at the board of decision of a great university, or sitting quietly, listening in characteristic attentive manner to the status of a search for a new professor at the medical school or the statement of the problems concerning the formulation of a policy of importance to our national security. We observe him, then, giving his opinion, couched always in simple, clear, incisive manner, and only when he believes that he has something of value to contribute. We see him, best of all, at the helm of his boat on the waters off Cape Cod, eyes fixed on the far horizon, carrying out expertly the maneuvers that only a gifted sailor can achieve after long years of practice. What new branches of medicine or science is he thinking about? What new territory is he planning to explore? For what new crisis is he preparing a program of action? We cannot read his mind, but of this we can be certain—whatever he is planning, the goal will be a worthy one, the territory unexplored, and the course not fully charted; there will be a challenge worthy of the man, and his contributions will be as notable as his leadership is unobtrusive.

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Observations Including a Measure of Its Concentration in Certain Locales

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It has been known since the turn of the century [1] that a heterologous antiserum to kidney tissue ("nephrotoxic" serum) when injected intravenously into an animal of the donor species can produce renal disease. Although at first this disease was not recognized as a glomerulonephritis, largely owing to contaminating hemagglutinins and hemolysins, later studies, particularly by Wilson and Oliver [2] and subsequently by Masugi,[3] established the unique localization and character of the lesion. It is now known that such antisera affect the kidney only and more specifically the glomeruli. Furthermore, it is now recognized that such sera are not strictly species-specific but are capable to a limited extent of cross reactions, as between rat and mouse,[4] rabbit and dog,[5] and man and dog.[6] Thus, in the case of the latter, rabbit anti-human-glomerular-basement-membrane antiserum is capable of producing an acute glomerulonephritis in the dog.

Even organ specificity no longer pertains, in terms both of the components of the kidney and of the organ as a whole. It was thought that the renal cortex only contained the requisite antigen(s)[7,8] and that it was localized to the glomerular capillary basement membrane.[9] It has now been shown that the renal medulla is likewise capable of producing a "nephrotoxic" antiserum.[4] Moreover, it has become clear in recent years that while nonrenal antiorgan heterologous antisera are, perhaps with few exceptions, ineffective in producing lesions in the target or other nonrenal organs of animals of the donor species, nevertheless, they are capable of producing glomerulonephritis in these animals. This has been demonstrated to be the case for such heterologous antisera developed against muscle,[10,11] retina,[12] lung,[4,11,13,14] placenta,[4,15-17,19] aorta,[20] brain,[11] liver,[11] stomach,[21] intestine,[4] and heart.[4,22] Earlier studies, too, by Smadel and Farr [18] indicated the possibility of a common "nephrotoxic" antigen(s) in nonrenal organs or tissues. Thus, the production of a glomerulonephritis in the rat with rabbit anti-rat-renal antiserum could be modified or annulled by prior absorption of the antiserum with rat liver.

Recent studies have been concerned with an attempt to analyze the complexity of antigenic materials in any tissue and the corresponding pattern of antibodies in heterologous antisera. A brief review follows of some of these studies as they may relate to the "nephrotoxic" antigen(s).

Gamma-globulins from a variety of antiorgan heterologous antisera have been labeled with radioactive isotopes and their distribution traced in animals of the donor species after intravenous injection.[23,24] It has been shown that there is preferential localization of some of these antibodies to certain organs. By elution and reinjection of the antibodies localized to such organs and by in vitro observations on the binding by organ homogenates of the labeled antiorgan antibodies a degree of organ specificity of these antibodies could be demonstrated. Pressman and his co-workers [25-27]

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have stressed the time factor in localization. Antibodies which localize rapidly presumably do so on the vascular bed or in a structure that is in very close contact with the circulation. They have assumed that there is more than one such rapidly localizing antibody against any particular organ. On the other hand, Bale, Spar, Goodland, and Wolfe,[28] as well as Pressman,[23] have cautioned that in the interpretation of the specificity of such localizing antibodies the following factors have to be taken into account: the mode of injection, the relative rate of circulation through the organ, vascular permeability to proteins, intra- or extracellular location of the antigens, and, finally, the organ specificity of the antibodies.

However, in the rapid and preferential localization of antiorgan heterologous antibodies, the kidney has been frequently involved and radioautographs in the few instances when they have been reported have demonstrated a concentration of the radioactive label within the renal glomeruli. In keeping with this have been the observations of Mellors, Siegel, and Pressman[29] that the γ-globulin of rabbit anti-rat-kidney and anti-rat-lung antisera could be demonstrated in rat renal glomeruli following intravenous injections of these antisera by applying fluorescently tagged chicken anti-rabbit-γ-globulin antibodies to sections of the kidney.

Other experimental procedures have attempted to analyze the organ or tissue antigens by in vitro methods. Cruickshank and Hill,[30] by the use of Coon's technique with fluorescently labeled rabbit antibodies to homogenates of rat kidney, lung, and renal glomeruli, have demonstrated common antigens in reticulum, basement membranes (widely including glomerular capillary basement membranes), neurilemma, and sarcolemma of the tissues of the rat. Scott,[31] using the same technique but with rabbit antibodies to human glomeruli and synovial membrane, respectively, suggested that the renal glomerulus contained at least two antigens. One of these present in glomerular basement membranes was shared by vascular and nonvascular basement membranes in other organs. The other was more widely distributed and found in much the same sites as argyrophilic reticulum. Goodman, Greenspon, and Krakower[32] postulated on the basis of serologic agglutination and absorption tests with the acellular membranes of glomeruli and stroma of other tissues that glomerular capillary basement membrane may possess at least three types of antigen. One is responsible for the production of a "nephrotoxic" serum. A second is related to renal tubular basement membrane, and a third, to the collagens, such as in tendon and cornea. Employing somewhat similar agglutination and absorption tests with rat and human material and a somewhat wider range of tissues for absorption, both fresh and enzymatically and chemically treated, Milazzo[33] agreed with the findings of Cruickshank and Hill. He supported the view that antibody to reticulum was the major active constituent of "nephrotoxic" sera.

In the extensive studies on the "nephrotoxic" antigen(s) by Baxter and Goodman,[4] rabbit antirat sera were developed against a large number of rat organs; 0.5 gm. or at times 1.0 gm. quantities of homogenates of these organs without adjuvant was injected twice weekly for three weeks. After a period of rest, the immunization procedures were repeated at least twice again. They found that renal medulla, lung, and placenta were capable of evoking a heterologous "nephrotoxic" serum with about the same potency as that of rat renal glomeruli. Other organs, such as intestine, heart, and stroma of liver, were much less capable of doing so; while ovary, brain, muscle, spleen, adrenal, and liver failed to produce a "nephrotoxic" serum. They also absorbed a standard rabbit anti-rat-kidney antiserum with fixed and to some extent graded quantities of homogenates of various whole tissues of the rat and tested the nonabsorbed portion of the serum for its "nephrotoxic" activity in rats. There was fair agreement between these observations

and those of the direct biologic testing of the antisera. These results suggested that the "nephrotoxic" antigen(s) was probably present in the stroma of organs, varying in content from one organ to another and to some extent independent of the total content of blood vessels and other supporting tissue present in the organs. They felt that it was possible that the content of the antigen in various tissues might be related to a specific anatomic component of the stroma which had not been clearly defined.

Some attempts[34-38] have been made to isolate and identify the "nephrotoxic" antigen(s) chemically, but that has not been achieved to date.

While the "nephrotoxic" antigen(s) has been localized to the basement membrane of the renal glomerular capillaries,[3] the summary of the above recent observations points toward but does not establish an extension of this finding to the capillaries of other organs, as suggested by Spühler, Zollinger, and Enderlin[11]; Rother,[39] and Mellors, Siegel, and Pressman.[29] In fact, these recent studies have introduced an element of confusion. It is clear that antigens in common with the "nephrotoxic" one are to be found both in renal glomerular capillary basement membrane and elsewhere, particularly in structures related to the broad group of reticulins as defined by Robb-Smith.[40] Whether these structures other than capillaries contain the "nephrotoxic" antigen is undetermined.

Likewise, with reference to the localization of the heterologous antiorgan antibodies, no distinction is possible as to which of the complex of antibodies produced against whole organ homogenates localize in or near vascular beds. It is unknown whether any of these are related to the specific "nephrotoxic" antibodies. In view, however, of the unique location of the disease produced by these antiorgan antibodies, primarily within the renal glomerulus, it would follow that the demonstrated antibodies in this organelle must include the specific "nephrotoxic" ones. The question remains, however, why this unique localization of the disease? Why are not vascular or nonvascular tissues affected elsewhere? In that regard, Zollinger[41] has suggested that hemodynamic factors, namely, the large volume of blood flowing through and the high filtration rate of glomerular capillaries account for the localization of the disease to the glomerulus. Rother[39] has suggested that the glomerular capillaries are immunobiologically different from capillaries elsewhere. However, the findings of Baxter and Goodman[4] fail to support distinctive quantitative differences in the "nephrotoxic" antigenic content of glomeruli and have pointed toward a similar content in other organs, such as lung and placenta. Neither have they lent support to any qualitative differences between the specific antigen(s) in the glomerulus as compared with other organs.

The present experiments attempt to clarify some of these problems.

Procedures and Methods

A. Determination of Presence of "Nephrotoxic" Antigen(s) in Variety of Organs and Tissues

The organs and tissues were obtained from dogs as sterilely as possible. They were immersed in sterile isotonic saline and stored in the cold at —15 C. As a rule, they were allowed to thaw slowly at 4 C for 15 hours prior to use. The tissues were dissected under sterile conditions in a sterile cold room maintained at 5 C. At all other times, they were kept in sterile containers in a bath of ice.

1. *Preparation of Tissue by Frozen Sectioning for Sonic Vibration.*—Articular cartilage was obtained from elbow and knee joints. The epithelial layer of the cornea was removed by scraping the surface with a sharp scalpel blade while the eye was still in the frozen state. The cornea was then excised, and the inner epithelial layer was removed in the same way.

Heart valves included both the A-V valves as well as the semilunar cusps. The A-V valves were trimmed of grossly apparent musculature.

Tendon was stripped of its surrounding and enclosing sheaths, liberating the individual bundles. Ligamentum flavum obtained from the dorsal vertebral laminae was trimmed. Only the central, less vascular and more elastic portion was used. The more vascular and more fibrous portions to either side were discarded. The thoracic portion of the aorta in large part was used, including the cranial

abdominal portion. The adventitia was stripped away. The aorta was opened, trimmed to a rectangular shape, gently flattened, and measured. The number of vascular apertures was counted, and their dimensions, recorded. The sum of the areas of the vascular apertures was deducted from the total area. A plane of cleavage could be found in the media whereby the aortic wall could be roughly separated into an inner third and an outer two-thirds.

The posterior vena cava caudad to the liver was handled in the same way as the aorta except that no cleavage could be obtained and the whole wall except for the adventitia was used.

In the case of lung, the pleura was stripped or shaved away while the lung was still in the frozen state. Subpleural blocks of lung were removed and were used exclusively. These blocks were taken as close to the previous pleural surface as possible in order to exclude larger bronchial or vascular branches.

The choroid of the eye represented the portion posterior to the ora serrata and excluded the region of the optic disk.

The renal medulla was taken from the papillary portion well away from the corticomedullary junction, so that there could be no possible inclusion of juxtamedullary glomeruli.

In the case of the testis, the tunic was dissected away. The testicular substance was sliced into smaller fragments.

The material thus prepared for all the above tissues was washed with isotonic saline by gravity or centrifugation. The fragments were then shaved on a freezing microtome two, three, or more times until the smallest and thinnest possible sections were obtained. These were then repeatedly washed in isotonic saline solution by centrifugation. The washing was particularly thorough for testis in order to free the tissue of as much sperm as well.

These shaved and washed tissues were packed by centrifugation, drained of fluid, and weighed. They were then sonically vibrated in small quantities in a Raytheon magnetostriction oscillator, Model S-102 (Raytheon Mfg. Co., Waltham, Mass.) operated at 130 volts for 20 to 30 minutes. In order to free the tissue of cells, they were variously vibrated in 8% saline, 0.85% saline, or distilled water. Heart valves, lung, ligamentum flavum, and renal medulla were vibrated in sterile 8% saline. Testis, aorta, and vena cava were vibrated in sterile distilled water. All the others were vibrated in sterile isotonic saline. After vibration, all tissues were returned to and washed repeatedly in sterile isotonic saline by centrifugation. The sediments were packed by centrifugation at 1500 rpm for three to five minutes, drained, and weighed. They were always checked microscopically to be certain that all cells had been removed. The supernatant fluids of the vibrated tissues were collected and partially lyophilized to reduce the volume. In the case of those in which the vibrating fluid was isotonic saline, the material was tested in animals. The same was true of those vibrated in distilled water. NaCl was added to isotonic level. On the other hand, the fluids from those tissues vibrated in 8% saline could not be tested in animals, owing to the high concentration of salt. The danger of bacterial contamination in the course of dialysis discouraged us from using this procedure in order to eliminate the excess of salt. The estimated amount of material lost to the supernatant was obtained by subtraction of the amount of stromal sediment from the total amount of originally shaved and washed material.

2. Preparation of Tissues Not Necessarily Requiring Frozen Sectioning or Sonic Vibration.—The anterior and posterior surfaces of the ocular lens were trimmed to exclude the capsular nucleated portions. The lenses were then washed, packed by centrifugation, and weighed. They were homogenized in a Servall Omniblendor operating at 15,000 rpm and 115 volts. The blendor was chilled in a bath with chipped and cracked ice. The homogenized lens was used for animal inoculation.

In the case of heart, endocardium and epicardium were cut away. The myocardium was shaved with a scalpel blade. The shavings were thoroughly washed in isotonic saline. They were sonically vibrated in isotonic saline for 5 or 10 minutes. The coarse stroma of the heart separated out and aggregated into small clumps. These were removed. The striated muscle fibers broke up readily into fine particles no longer recognizable as striated structures. No nuclei or nuclear fragments were identifiable, however. The particles represented cytoplasmic granules. Spun at 3500 rpm, the sediment was tested for its "nephrotoxic" activity. The supernatant was spun at 15,000-18,000 rpm in a Servall centrifuge. The sediment and supernatant were also tested for "nephrotoxic" activity.

Endothelial cells were obtained from fresh and fresh-frozen thawed aortae by stretching the aorta and pinning its edges. The intimal surface was flushed with sterile saline to free it of adherent red blood cells, white blood cells, and platelets. It was then gently scraped with the long edge of a spatula. The scrapings were collected in isotonic saline. They were washed with saline by repeated centrifugation. Individual and sheets of endothelial cells so obtained were used for inoculation of rabbits.

In order to relate the surface area of large vessels and their very superficial nonvascularized intimal and immediate subintimal portions with their "nephrotoxic" activity, fresh-frozen and thawed aortae and venae cavae were trimmed to form a

rectangle. Their intimal surfaces were flushed with isotonic saline and flattened on paper toweling, and surface areas were determined. The areas of vascular orifices were deducted. In order to conserve material in the later phase of the experiment, areas were determined planimetrically without trimming the vessels. The toweling with the flattened vessels was then placed over a sterile metal plate set on a block of solid carbon dioxide (dry ice). The intimal surface of the vessels was scraped with the sharp beveled edge of a razor blade. Using the bevel as a guide, only the very superficial inner layer of the vessel was removed, so as to avoid the vascularized portion of the media. The scrapings were washed repeatedly in isotonic saline by centrifugation. They were used for inoculation of rabbits.

The choroid plexuses of fresh-frozen and thawed brains were removed from the fourth and lateral ventricles. They were placed in sterile Petri dishes covered with isotonic saline. The capillary-bearing portions of the plexuses were then excised under the dissecting microscope, with use of iris scissors and dissecting needles. All larger vessels, arterial and venous, and extraneous tissues were dissected away. The smaller arterial and venous branches intimately associated with the varied shaped and complex capillary tufts were unavoidably retained. This dissection was likewise performed in the sterile cold room at 5C. The plexuses were then repeatedly washed in saline, shaved on a freezing microtome, and tested for their "nephrotoxic" antigenic content, both as shaved fragments and as their acellular stromal components obtained by sonic vibration in 8% saline. The vibrated material was rapidly changed into isotonic saline and washed repeatedly by centrifugation. Each brain on the average yielded about 28.6 mg. of washed shaved capillary-bearing plexus.

The ciliary processes of fresh-frozen and partially thawed eyes were obtained as follows. The eyes were trimmed of all extraocular tissues. The corneae were excised. Lens and vitreous were gently extruded as soon as the eyes were adequately thawed. The eyes were then inverted and gently washed in isotonic saline. By use of stout forceps, inserted into the inverted cavity of the eye as holder, and immersing the eye in a shallow basin containing isotonic saline, the ciliary processes could be made to float. With a spotlight focused upon the eye, these processes could then readily be plucked with iris forceps. The processes were collected in a test tube with isotonic saline and were washed repeatedly to remove particles of melanin pigment by suspension and settling. They were packed by centrifugation and weighed. They were used in part as intact processes for biological testing or they were vibrated in 8% saline. The acellular stroma with predominantly liberated pigment granules was washed in isotonic saline and tested biologically as well. The preparation of the ciliary processes was performed under sterile conditions in the cold sterile room. On the average, 3.7 up to 4.0 mg. of ciliary processes was obtained per eye.

Renal glomeruli were isolated in a manner described previously.[9] By the careful use of silicone-coated containers, the counts were made with a hemacytometer. Isolated renal glomeruli were vibrated in isotonic saline in order to obtain glomerular basement membrane.

3. *Procedure for Giving Inoculations to Rabbits and for Subsequent Testing of Antiserum in Dogs.*—In the course of obtaining the tissue products for biological testing, weighings were made of the tissue prior to shaving on the microtome and after repeated washing, packing, and draining of the shavings. In like manner, the acellular stroma after sonic vibration was washed in isotonic saline, packed by centrifugation, drained of excess fluid, and weighed. Aliquots of the tissue were removed for the determination of fat-free dry weight and nitrogen content. The materials were reweighed, suspended in small amounts of isotonic saline, mixed with equal parts of an aluminum jelly prepared by the method of Tracy and Welker,[10] and injected in known amounts into the intramuscular tissues of rabbits. The rabbits were bled three weeks later. The sera were stored at −15 C. Prior to use, they were rapidly thawed. Dogs were given intravenous injections, with use of 1.5 cc. of the test serum per pound of dog. The dogs were killed seven days after injection. The urine was tested for protein. Complete autopsies were performed, and histologic sections of all organs were examined microscopically.

For any given tissue and for any given graded amount of the tissue, at least three and often many more rabbits were given inoculations. The serum of each of these rabbits was tested in individual dogs. Sera were not pooled.

As a rule, in testing tissues for their "nephrotoxic" content, the following approximate amounts on a wet weight basis were employed: 25, 75, 200, and 500 mg. and 1 and 2 gm., respectively. These higher values were exceeded or not tested if positive tests were obtained at lower levels. The level at which 60% or more of the sera gave positive tests for glomerulonephritis was regarded as the basic amount of tissue which contained the equivalent of a unit of "nephrotoxic" antigen(s). A positive test was one in which the dog developed some degree of proteinuria within the seven-day period and where there were glomerular lesions, even if these were focal in character. In strongly positive tests, there was abundant protein in the urine and the kidneys presented the classical gross

and microscopic features of acute hemorrhagic glomerulonephritis. All glomeruli were affected.

B. Determination of Approximate Volume and Area of Capillary Bed of Certain Selected Tissues

In all instances, dogs were heavily sedated with pentobarbital (Nembutal) (0.5 cc. per pound of a 3% solution) administered intravenously. This was followed by an intravenous injection of heparin (2.0 mg. per pound of dog) dissolved in isotonic saline.

Renal Glomeruli.—Both renal arteries and veins were cannulated and perfused with warm Ringer-Locke solution. This was delivered from a cistern by gravity at a pressure of 130 mm. Hg. When the perfusate was clear, the perfusion fluid was changed to that of a commercial yellow latex (General Biological Supply House, Inc., 8200 S. Hoyne Ave., Chicago 20) diluted with Ringer-Locke solution volume per volume. It was necessary to filter the diluted latex through packs of gauze in order to free it of gross particles. There was very little flow of latex through the renal vein. The tubing leading to the cannula was clamped. The kidneys were gently removed and placed in the cold at —15 C. Some time later, they were slowly thawed overnight at 4 C and processed for glomeruli in the same way, as previously described, for the normal kidney.[9] The isolated glomeruli suspended in isotonic saline varied in their content of latex. This variation could readily be detected in a strong light source with the naked eye. The well-filled glomeruli were, therefore, hand picked with a pipette. After a satisfactory number of glomeruli had been so collected, samples were taken for counts and for the determination microscopically of the per cent of well-filled glomeruli. Measurements of the capillary diameters were made. The glomeruli were then packed by centrifugation and weighed. They were dried in an oven to constant weight after lipid extraction with alcohol-ether. The yellow dye was then extracted in the dark by treating the dried latex-filled glomeruli with *o*-dichlorobenzene (1-2-dichlorobenzene, B. P. 179.5-180.5).

Frequent changes of *o*-dichlorobenzene were made until no further dye could be extracted. The extracts were collected and saved for spectrophotometric determinations. The glomeruli were dried to constant weight again. They were then digested with hot 30% KOH for 20 to 30 minutes. The residue was washed repeatedly with distilled water by centrifugation and then dried to constant weight.

Control uninfiltrated glomeruli and accurately measured volumes of latex were treated in the same way simultaneously. From seven latex-infiltrated kidneys, 2,665,000 glomeruli were obtained. Of these, 93.3% showed 80%-100% filling; 3.6%, from 50%-70% filling; 2.0%, from 20%-40% filling, and 1%, from 0%-10% filling. The glomerular preparation microscopically otherwise contained few contaminants. There were only 5.6% tubular fragments, while 40.4% of the parietal capsules of the glomeruli were retained.

In place of commercial latex, in order to obtain better capillary filling, the following latex mixture was used for the organs or tissues described below. Natural latex 62.6% (Chicago Latex Products, 3019 W. Montrose St., Chicago 18) was diluted with Ringer-Locke solution volume per volume; 50 cc. of a saturated solution of a yellow dye (Rubber Yellow G. L., Code YL-632-Dr, E. I. duPont de Nemours and Company, Wilmington, Del.) in Ringer-Locke solution was added to every 4 liters of the mixture of natural latex with Ringer-Locke solution. The mixture was filtered repeatedly through packs of gauze prior to use. The fine particulate quality of the suspension was checked microscopically.

Lung.—With the dog prepared as described above, a cannula was inserted into the pulmonary artery. A second cannula was inserted into the left auricle of the heart. The venae cavae were tied. Warm Ringer-Locke solution was perfused through the cannula in the pulmonary artery by gravity at a pressure of 130 mm. Hg. When the perfusate from the left auricle was clear, the latex was infused by gravity at the same pressure. The latex perfusion was continued until a good brisk flow was obtained from the auricle. Samples of the effluent were collected. The tubing to the cannulae was then clamped. The lung, on perfusion, became somewhat inflated but failed to fill the pleural space. The animal was moved into a cold room at —10 C. The frozen latex-filled lungs were removed and stored in the Deepfreeze at —15 C. For determination of the volume and area of the vascular bed, the lungs were removed and while in the frozen state the pleura was trimmed away. Thin subpleural strips of lungs were removed. Samples of these were examined microscopically for the completeness of capillary filling and to determine the vascular diameters. These strips were washed, dried, defatted with alcohol-ether, dried to constant weight, extracted with *o*-dichlorobenzene, and digested with KOH, as with the renal glomeruli. Careful subpleural shavings of frozen Ringer-Locke-perfused lung were treated in the same way, as were accurately measured and weighed samples of the latex mixture, both of the influent and effluent.

Choroid Plexuses of Brain and Ciliary Processes of Eyes.—Dogs were prepared as previously. The internal carotid arteries were cannulated, and perfusion was started with warm Ringer-Locke solution at 130 mm. Hg. by gravity. The jugular veins

were then cannulated. Once a good flow was established, both vertebral arteries were ligated near their origins. The trachea and esophagus were then severed and retracted caudally. The atlantooccipital ligament was exposed and incised. The basilar artery and the entering vertebral arteries were brought into view, and all three vessels were clamped with silver McKenzie clips. The perfusion with Ringer-Locke solution was continued until the perfusate from the jugular veins was clear. The latex was then injected through one internal carotid artery, with use of a circulating pump (Model A-I, Eastern Industries, Hamden, Conn.) operating at a pressure of 150 mm. Hg. When the latex flowed freely through the jugular vein on the same side, the perfusion was stopped. The vessels were clamped. The procedure was then repeated on the opposite side. Samples of the effluent were obtained. The dog was then frozen in a cold room at −10 C. Subsequently, brain and eyes were removed. The capillaries of the brain failed to fill. The latex-filled capillary-bearing portions of the choroid plexuses and the ciliary processes were removed and dissected in the sterile cold room at 5 C in the manner described elsewhere. In addition, each ciliary process was checked under the dissecting microscope for the completeness of the capillary filling. Only those completely filled and those filled portions of the ciliary processes were included. The nonfilled portions were excised with iris scissors or teased away with dissecting needles. Unfilled ciliary processes were discarded. The samples of choroid plexuses and ciliary processes from 24 dogs were processed as in the case of lung, including control uninfiltrated samples of the same tissues. Accurately measured and weighed, samples of the latex and its effluent from the jugular veins were likewise processed. Vascular diameters were determined from mounted samples of the latex-filled chorioid plexuses and ciliary processes.

Methods to Compute Vascular Volume and Area of Vascular Bed of Sampled Tissues.—In order to determine the vascular volume of the latex-infiltrated tissues which were sampled, the following simultaneous linear equations were used. Of the two variables used in these two equations, one could be eliminated by subtraction.

Let X represent the fat-free dry weight of the tissue in the latex-infiltrated sample and Y, the fat-free dry weight of the latex in the same sample.

EQUATION 1.—$X+Y$=the fat-free dry weight of the whole latex-infiltrated sample.

If Factor A represents the fraction of dry weight of tissue left after KOH digestion of the dried fat-extracted uninfiltrated control sample of washed or washed perfused tissue and Factor B represents the fraction of dry weight of latex left after KOH digestion of the measured, weighed, fat-extracted, and dried sample of the influent, then

EQUATION 2.—$AX+BY$=the dry weight of the latex-infiltrated sample after KOH digestion.

$A+B$ could be determined from the control tissue and latex samples, respectively. Accordingly, the values for X and Y could be obtained.

From these values one could determine the weight of rubber per milligram of fat-free dry tissue. Relating the dry weight of rubber to the measured volume of the sample of latex used as an influent, one could then determine the volume of latex per milligram of fat-free dry tissue. Since in the case of lung, choroid plexuses, and ciliary processes, the effluents contained approximately the same amount of dry latex per unit volume as the influent, there was neither a factor of dilution nor of concentration of the latex as it traversed the capillary beds of these tissues. The volume of latex per milligram of fat-free dry tissue was therefore a valid measurement of the vascular capacity. Although no effluent was obtained in perfusion of the kidneys with latex, it may be assumed from the total dry weight of the sample of 27.9%, as compared with 25% to 27% in varied samples of infiltrated lung and 19% for choroid plexus and ciliary processes, that there was probably little concentration of the latex as a result of filtration in the glomeruli. This high per cent of dry weight compares favorably with the approximate 25% dry weight or more of the latex samples.

The values for vascular capacity obtained by the above gravimetric method were checked with the spectrophotometric determinations of the concentration of the fully extracted yellow dye from the latex-infiltrated tissue samples. The dye extracted with *o*-dichlorobenzene from the measured volume of latex used for perfusion was used as a standard. These extracts were yellow crystal-clear solutions. All necessary dilutions were made with *o*-dichlorobenzene.

In order to determine the area of the vascular bed corresponding to 1 mg. of fat-free dry tissue, the following formula was used. It was based on the assumption that vessels can be regarded as cylinders. Maximal, minimal, and the most frequent or mean diameters were used in the estimation of the vascular area.

$\frac{2 \times V \times 10^{12}}{r}$=area of vascular bed/mg. fat-free dry tissue in μ^2

where

V=vascular volume per milligram of fat-free dry tissue in milliliters

r=radius of vessel in μ

Results

Comparison of "Nephrotoxic" Antigenic Content of Avascular, Poorly Vascular, and

Vascular Tissues.—The cellular elements of organs have not been implicated in the search for the source of the "nephrotoxic" antigen(s). This was clearly shown to be the case for renal glomeruli where the endothelial cells and visceral and parietal epithelial cells were excluded from consideration.[9,43] In the current study, since blood vessels were suspect as the source of the antigen(s), endothelial cells were removed from dog aortae. Two samples of 43 mg. each and one of 104 mg. were injected into rabbits. The three antisera were ineffective in producing glomerulonephritis in the dog. In order, therefore, to reduce the complexity of antigens in organs and tissues for the purpose of this study, all cells were removed by sonic vibration. This was not necessary in the case of the lens of the eye, except for the immediate capsular aspects which were removed, and was unavoidable in the case of the myocardium. The effectiveness of the exclusion of cells was checked by histologic examination of the residual stroma. The varied use during vibration of 8% saline, isotonic saline, or distilled water for the different tissues was based on the effectiveness of one or other of these to remove all apparent cellular components. The antisera to these stromal elements always brought out the "nephrotoxic" antigen(s) more readily and clearly than when the whole tissue was used. It was for that reason that highly cellular parenchymatous organs, such as liver and spleen, were purposely avoided, since it was felt that it would be very difficult to obtain an acellular stroma from them.

The problem was to determine what component of the stroma contained the "nephrotoxic" antigen(s). Accordingly, a

TABLE 1.—*The "Nephrotoxic" Antigenic Content of Canine Tissues*

Type of Tissue	Wet Wt. of Stroma, Gm. *	Dry Wt. of Stroma (Nitrogen Content), Mg.	Tissue in Supernatant, Gm. †	Results ‡	
				Stroma	Supernatant
A. Avascular					
Articular cartilage	0.927 (1.522)	101.0 (10.1)	1.5	—	—
Cornea	4.0 (5.788)	326.8 (23.8)	0.73	—	—
Lens	10.0 (10.0)	1,003.0 (150.4)		—	—
B. Poorly vascular					
Heart valves	1.0 (1.6)	45.0	0.575	—	—
Tendon	3.8 (4.8)	380.0 (27.4)	0.475	—	—
Ligamentum flavum	1.2 (1.37)			—	
Inner third of wall of aorta	5.0 (7.1)	455.0 (37.3)	2.88	—	—
Outer two-thirds of wall of aorta	1.2 (2.7)	118.8 (12.5)	1.0	—	—
C. Well vascularized					
Myocardium	0.5 (0.648)	48.5 (4.6)	0.5	+	—
			0.1 (high-speed centrifugate)		(including high-speed centrifugate)
Lung	0.45 (2.07)	44.0 (4.1)	1.0	+	+
Vena cava	1.0 (1.232)	96.0 (9.4)	120.6 mg. solids ♯ with 1.7 mg. nitrogen	+	—
Renal medulla	0.175 (0.351)	19.9 (1.9)	1.5	+	—
Testis	0.5 (1.053)	34.6 (3.8)	2.0	+	—
Choroid of eye	1.0 (2.079)	176.0 (13.0)		+	
Choroid plexus of brain	0.25 - 0.887 (0.508 - 1.841)	23.5 - 93.9 (1.6 - 8.5)		+	
Ciliary processes of eye	0.486 (2.375)	58.3 (6.2)		+	
Renal glomeruli	0.005 - 0.01 (0.015 - 0.025)	0.17 (0.02) Average	0.125-0.150	+	+

* Equivalent weight of washed or shaved washed whole tissue. The values listed represent the maximal amount tested in those which gave negative results.

† Estimated amount vibrated tissue, cellular components predominantly in supernatant.

‡ A positive result implies that 60% or more of the rabbit antisera to the particular tissue at the stated amount produced glomerulonephritis when tested in dogs.

♯ Estimate of wet weight of solids in the supernatant could not be obtained because of increase in weight of the stroma by vibrating the vena cava in distilled water.

number of tissues were tested of varying stromal composition and with varied degrees of vascularity.[44]

It is apparent from Table 1 that the three avascular canine tissues, namely, cartilage, cornea, and a homogenate of the lens, were seemingly devoid of the antigen(s). Every antiserum developed in the rabbit to these tissues failed to produce glomerulonephritis in the dog. The highest levels that were tested were equivalent to or greatly exceeded the positive values in vascular tissues.

Poorly vascular tissues, such as heart valves, tendon, ligamentum flavum, and the media of the aorta, all failed to yield 60% or more positive antisera at all levels, including those equivalent to or exceeding the positive values in vascular tissues. However, there were occasional positive sera from stroma and supernatant at the different levels tested, but there were never enough such sera at any level to constitute 60% or more of the total. It would, therefore, appear that there was some "nephrotoxic" antigen(s) in these poorly vascular tissues but apparently in low concentration.

On the other hand, well-vascularized tissues, such as myocardium and the acellular stromata of lung, chorioid plexus, choroid of the eye, ciliary processes of the eye, renal medulla, testis, and the fairly vascular media of the vena cava as well as renal glomeruli, all yielded 60% or more positive antisera at the levels indicated in Table 1. Thus, 1 gm. or less of acellular stroma of these canine vascular tissues contained enough "nephrotoxic" antigen(s) to produce a significant number of positive rabbit antisera. Even as in the case of sonically vibrated renal glomeruli, a certain amount of the antigen(s) was lost to the supernatant. Accordingly, the supernatants of these positive tissues contained enough antigen(s) to give positive sera in instances but only in a significant number in the case of lung at the level indicated in Table 1.

Surveying the known histologic compositions of the tissues that were tested, it is apparent from Table 2 that neither collagen nor elastin was likely to be the source of the "nephrotoxic" antigen(s). Both were present in tissues with little or greater amounts of the antigen. Thus, cornea, tendon, and renal medulla each contained over 90% collagen on a dry weight basis after digestion with N/10 NaOH. Yet, cornea had no apparent antigen; tendon had very little; whereas renal medulla was well supplied with it. Similarly, aorta with little antigen contained approximately 50% elastic tissue, whereas lung, which was antigenic, contained 35.8%. Reticulum associated with the smooth muscle of the media of the aorta, a poorly antigenic tissue, was also associated with the vascular fibers of the myocardium and alveolar septa of the lung, tissues very rich in the antigen. Epithelial basement membranes, although present in antigenic tissues, were absent from tissues which were similarly antigenic. Although the specific interstitial matrices, such as those of hyaline cartilage and cornea, were

TABLE 2.—*Content of Collagen and Elastin in Acellular Stromata of Canine Tissues Expressed as Per Cent of Dry Weight After N/10 NaOH Treatment* *

Type of Tissue	Collagen	Elastin
A. Avascular		
Articular cartilage	83.8	8.1
Cornea	93.9	1.3
Lens †	32.5	53.9
B. Poorly vascular		
Heart valves	84.6	12.4
Tendon	93.2	5.2
Inner third of wall of aorta	27.3	52.1
Outer two-thirds of wall of aorta	28.8	47.5
C. Well vascularized		
Myocardium ‡	0	0
Lung	55.0	35.8
Vena cava	69.7	18.6
Renal medulla	91.3	5.4
Renal glomeruli	76.7	11.6

* Determinations were made by the method of Lowry, O. H.; Gilligan, D. R., and Katersky, E. M.: *J. Biol. Chem.* 139: 795, 1941.

† 95.5% of the wet weight of the homogenate of the lens was dissolved by the N/10 NaOH. The values represent the findings in the remaining 0.5% of the wet material.

‡ All of the myocardial residue was dissolved by the N/10 NaOH. This residue represented fine particles derived from the striated muscle fibers. The coarse stroma aggregated in the sonic vibrator and was removed.

largely removed by sonic vibration, leaving in the main fine fibrils of collagen, nevertheless, enough of the matrix coated the residual fibers so that if it were of significance it would have been found in the more antigenic tissues. Even with the realization that the chemical composition of less specific interstitial matrices or so-called ground substances varies in different sites, there was no evident known variant common to the antigenic tissues which was not present in the nonantigenic ones with one exception, namely, that associated with the vascular bed.

As indicated above, the elastic, fibrous, and reticular tissues of the walls of blood vessels would not be expected to be the site of the "nephrotoxic" antigen(s) were they chemically comparable to that of similar nonvascular tissues. The poor antigenic qualities of the wall of the aorta as compared with the wall of the vena cava could hardly be ascribed to the different proportions of these elements (Table 2, for collagen and elastic tissue). It would more likely have to be ascribed to the richer small vascular bed or basically the capillaries of the wall of the vena cava as compared with that of the aorta. This is in keeping with the observation that tissues with abundant "nephrotoxic" antigen(s) had a rich capillary bed.

That capillaries and pre- and postcapillaries are the logical site of the antigen(s) gains support from two observations.

In the first place, recent electron microscopic studies have clearly established the presence of a very distinctive membrane beneath the endothelial cells of capillaries and sinusoids[45-62]; similar to that of glomerular capillary basement membrane.[63-74] This basement membrane is homogeneous in character, with often zones of different electron density. No fibrillar structures have been identified within it. The electron microscopic observations of Berrian,[75] on the aorta of the rat; of Fawcett,[46] on the arteries of the testis, and of Moore and Ruska,[47] on small arteries generally point to the absence of such a subendothelial basement membrane in small and large arteries. In these vessels, the endothelial cells are in contact, with the elastica interna sending cytoplasmic extensions through its fenestrae. On the other hand, the characteristic capillary basement membrane does appear to underlie the endothelial cells of terminal arterioles or metarterioles. On the venous side, no submicroscopic studies were found relating endothelial cells to its adjacent tissues. In view, however, of the irregular fine elastic fibers known from light microscopy to be beneath the endothelium, there is reason to believe that here too, at least in veins with an adequate musculature, there may not be a distinctive subendothelial basement membrane and the endothelial cells are in contact with elastic or possibly collagenous fibers. It may be assumed, however, that the subendothelium of postcapillary venules has the structural qualities of capillary basement membrane.

Secondly, it has been firmly established that the "nephrotoxic" antigen(s) is an integral part of the glomerular capillary basement membrane. By removing all the cells from isolated glomeruli by sonic vibration[9] or by their treatment with 60% (weight per weight) trichloracetic acid,[43] it could be shown that practically all of the "nephrotoxic" antigenic content of such glomeruli was retained in the naked capillary basement membranes.

With the compelling evidence, therefore, that the basement membrane of capillaries and pre- and postcapillaries are the site of this specific antigen(s), it became imperative to compare the volume of the capillary bed or better still its area in the different positive tissues that were tested. For, by so doing, under the controlled rigid biologic conditions of testing for the antigen(s), a measure of its concentration in these different tissues could thus be obtained. It was clear that despite every effort that was made in preparing the tissues to exclude large vessels as well as cellular components, as is clearly detailed in Procedures and

Methods, a comparison of the antigenic content of these tissues could not be made on the basis of weight. The small amount of glomerular capillary basement membrane, namely, 5 to 10 mg., necessary to produce positive antisera, was out of all proportion to that of other tissues. However, there was relatively little extraneous material associated with the preparation of glomerular basement membrane, while such material was of varying abundance in the others. Thus, there was extremely abundant melanin pigment in the form of granules, which could not be eliminated from the preparations of the choroid of the eye or of the ciliary processes. There were countless cytoplasmic granules from the striated muscle fibers in the preparation of myocardium. There was a large component of tubular basement membranes in the stroma of renal medulla and testis and of elastic and fibrous tissue in such structures as lung and vena cava.

It was only by knowing the area of the capillary bed per unit of tissue that one could logically compare the concentration of "nephrotoxic" antigen(s).

Capacity and Surface Area of Vascular Bed of Renal Glomeruli, Lung, Choroid Plexus, and Ciliary Processes.—The methods we finally employed to obtain values for the vascular capacity of these four tissues are given in the Procedures and Methods. The choice of these tissues was, however, not entirely by election. Anyone who has tried to fill a vascular bed completely must know that it is almost impossible for most tissues. If extravasates and the completeness of the filling are to be rigidly checked microscopically and if selected portions of the tissue are to be used to exclude larger vessels, one must employ a matrix which can be firmly set. After various trials with materials like Carbowax and different types of plastics, we found that latex served our purpose best. It could be set readily in the cold, avoiding unnecessary chemical agents. Synthetic latex as a commercial product was found to be best for renal glomeruli, since there was little flow beyond the efferent arterioles, making it possible to isolate the rubberized glomeruli. Natural latex was used for the others. It was found to enter smaller vessels more readily. It also could be combined with plasma proteins without coagulating the rubber, whereas the synthetic rubber was coagulated. Nevertheless, the high content of ammonia necessary to stabilize the latex, natural or synthetic, automatically served as a powerful vascular irritant. It is generally agreed that to obtain any degree of filling live tissues in most instances must be used. Consequently, despite combinations of the natural latex with Ringer-Locke solution and the addition of powdered plasma proteins in the proportion that they are found in blood and despite the use of vasodilators, the capillary bed could not be filled in its entirety or even at all except in the above four tissues.

It is of interest that of the tissues we used in our attempts to fill the vascular bed, namely, heart, skeletal muscle, testis, brain, and kidney (for an evaluation of the vascular volume of the medulla), the ones with which we had success represented in a sense filtering organs in three (renal glomeruli, choroid plexus, and ciliary process) and a low-pressure system in the fourth (lung). It is as though the vessels leading into the capillary beds of these tissues were ever so much less irritable than those of other organs.

The dye of the commercial latex at no time diffused out of the capillaries. Accordingly, it was possible to compare the results of the gravimetric analysis of the rubber content of the latex-infiltrated glomeruli with the spectrophotometric value obtained by extraction of the dye from the glomeruli with *o*-dichlorobenzene. The spectrophotometric value was 8% less than the gravimetric one. However, in the case of the natural latex with dye added, the latter did diffuse out of the vessels on occasions. The spectrophotometric values, accordingly, were 25.6% up to 76.6% greater than the gravimetric ones. The values for the capillary volumes of the four

TABLE 3.—*Approximate Capacity and Area of Capillary Bed per Milligram of Fat-Free Dry Tissue and Total Capillary Area for Amount of Tissue Necessary to Produce a Significant Per Cent of Positive Antisera*

	Capillary Bed/ Mg. Dry Tissue, Vol $\times 10^9$ cu. μ	Capillary Bed/ Mg. Dry Tissue, Area $\times 10^9$ sq. μ *	Capillary Bed for Pos. Antisera, Area $\times 10^9$ sq. μ †
Renal glomeruli	9.0	4.2 (0.77-8.6)	10.5
Lung	4.3	2.0 (0.09-2.7)	410.8
Choroid plexus	3.0	0.94 (0.09-1.9)	66.4-240.5
Ciliary processes	3.7	0.44 (0.20-3.5)	124.3

* This represents the area as determined from the diameter of the capillary vessels most frequently encountered in the latex-infiltrated samples. The extreme ranges are given in the parentheses and are derived from the vessels with maximum and minimum diameters respectively.

† Area of capillary bed for total amount of dry tissue essential to produce positive antisera.

tissues in Table 3 are therefore based on the determination of the weight of rubber in the samples after appropriate digestion of the tissues with hot 30% KOH.

It can be seen from Table 3 how much greater was the vascular volume of the renal glomerulus per milligram of dry tissue than that of the other three tissues. Lung, ciliary processes, and choroid plexus had decreasing volume per milligram of dry tissue, in the order given. The differences between these were not very great. The differences were more striking when the areas were determined. Even within the capillary tufts of the latex-filled glomerulus, the differences in diameter of the primary divisions of the afferent arteriole and the vessels meeting to form the efferent arteriole with those of the peripheral capillaries were appreciable. They varied from 46.8μ to 4.2μ. However, the most frequent capillary diameter was 8.6μ. In the latex-filled ciliary processes, despite the fact that all vessels are capillaries, their distensibility was such that they varied from 4.2μ to 74.4μ in diameter, with the most frequent capillary diameter being somewhere between 16.6μ and 51.1μ. In the latex-filled chorioid plexus, the commonest capillary diameters were between 8.5μ and 17.0μ; the smallest was 6.4μ. However, the maximum diameter of the included smaller arteries and veins did not exceed 127.6μ.

In the case of the immediate subpleural portion of latex-infiltrated lung, the vast capillary bed had a minimum of 6.3μ, in diameter, an average of 8.5μ, while the largest arterial or venous branches did not exceed 178.7μ. In both choroid plexus and lung, it was felt that the immense capillary bed so overshadowed the relatively few larger vascular radicles that the volumes and areas represented in effect a pretty close approximation to that of the capillary bed proper.

With use of the most frequent capillary diameter, the areas of these vascular beds were determined and are listed in Table 3. They varied from a minimum of $0.44\times 10^9\mu^2$, or 4.4 sq. cm. per milligram, of dry tissue for ciliary processes to $4.2\times 10^9\mu^2$, or 42 sq. cm. per milligram, of dry tissue for renal glomeruli.

To determine the total area of "capillary bed" necessary to produce a significant percentage of positive antisera for any of these tissues, one had only to multiply the given areas by the amount of fat-free dry whole-washed or shaved-washed and perfused tissue comparable to the amount of sonically vibrated material that was essential to produce these positive results. These values are listed in Table 3.

The range of values given for chorioid plexus was felt to be necessary. Significant numbers of positive antisera were obtained with 250 and 450-475 mg. of sonically vibrated plexuses, respectively. But these were weakly positive in the main. With 870-900 mg., however, better positive antisera were obtained.

It seems clear that if the biologic testing of the "nephrotoxic" antigen(s) is a measure of its concentration, then the basement membrane of glomerular capillaries has 10 to 20 times more of the antigen(s) (requiring 10 to 20 times less vascular area) to provide presumably an equivalent amount of the antigen(s) than choroid plexus or ciliary processes and 40 times more than the capillaries of the lung. It is of interest that there is such gradient. The glomerular capillaries with the highest concentration of the antigen(s) operate at a high hydrostatic pressure of more than 60 mm. Hg. The systemic capillaries with an intermediate concentration of the antigen(s), as represented by the choroid plexus and ciliary processes, operate at a balanced hydrostatic pressure of approximately 25 to 30 mm. Hg. Thus, in the case of the capillaries of the eye, although the intrinsic pressure is presumably about 50 mm. Hg, this is counterbalanced by an intraocular pressure of about 25 mm. Hg.[76] The capillaries of the lung with the lowest concentration of the antigen(s) have an intrinsic pressure of about 5 mm. Hg. These findings are in keeping with those reported previously,[77] relating the content of the "nephrotoxic" antigen(s) to changes in pressure within renal glomerular capillaries and to corresponding changes in tension within the capillary basement membrane. With increase in glomerular capillary pressure as with vena cava constriction above the level of the renal veins, there was an apparent increase in concentration of the antigen(s), whereas with compensatory renal hypertrophy with presumed increased blood flow and fall in pressure within the glomerulus, there was an apparent decrease in concentration of the antigen(s). Neonatal glomeruli operating at a lower pressure also contained less of the antigen than in the adult.

It would appear that in the organization of the capillary basement membrane in different locales there is a quantitative relationship between the structural materials that go to form the membrane and the pressure, flow, and filtration characteristics of the particular capillary bed. The "nephrotoxic" antigen(s) as an integral part of the membrane would be expected to share in these quantitative variations. It is possible that there may be a constancy in the structural organization of the basement membrane in different capillaries and that the variations noted here may be an expression of its thickness. The smaller surface area of glomerular capillaries would with a thicker basement membrane give rise to a total volume of membrane, or mass of membrane, equivalent to the larger surface area of pulmonary capillaries but with a thinner membrane. However, this difference in thickness of capillary basement membranes is not easy to establish in view of the different states of expansion of the capillaries and the insufficient data from electron microscopic studies of such measurements for different capillary beds in one species. Two examples are chosen for illustration. Measurements given or those that were made of the thickness of the basement membrane of some of the illustrated electron microscopic pictures of capillaries are as follows: In Hall's[67] illustration of rat glomerular capillaries, the thickness of the basement membrane varied from 0.19μ to 0.23μ, while in Low's[78] illustration of rat lung there was a variation in thickness of capillary basement membrane from 0.096μ to 0.16μ. Pease's estimate of the thickness of the rat glomerular capillary basement membrane was 0.1μ. In the case of the mouse, Yamada's[71] estimate of the thickness of glomerular capillary basement membrane was 0.08μ and that of Dalton,[65] 0.1μ; Karrer's[62] reproductions of electron microscopic pictures of the capillary basement membrane of mouse lung yielded estimates varying from 0.07μ to 0.22μ in thickness, while Ekholm and Sjöstrand's[58] estimate of the thickness of the periendothelial space in mouse thyroid was 0.15μ to 0.2μ. Hence, for the present, more information will be necessary before it can be ascertained whether in the case of glomerular capillaries, for example, there is more "nephro-

toxic" antigen(s) per unit amount of basement membrane, i. e., greater density of the antigen(s), or whether there is a greater amount of basement membrane per unit surface area with uniform density of the antigen(s). It seems more likely, however, that the differences in thickness of basement membrane will not be significant enough to account for the observed differences in antigenic content and that both variants but particularly density rather than the amount of membrane per unit surface area are at play in explaining these differences.

Subendothelium of Aorta and Vena Cava.

With a knowledge of the range of total capillary area which is necessary to contain an adequate amount of the "nephrotoxic" antigen(s) for positive biologic tests, the subendothelium of larger vessels was reexamined for its possible content of the antigen(s). The inner third of the wall of the aorta had yielded predominantly negative antisera where up to 5 gm. of sonically vibrated material had been used, derived from a surface area of as much as 103 sq. cm. The positive results with the wall of the vena cava were obtained with 1.0 gm. of vibrated material derived from a total surface area of 14 sq. cm. The superficial intimal scrapings of these two vessels were, therefore, tested. Care was taken to include the superficial elastic membranes, but every attempt was made to avoid the capillaries of the vasa vasorum. This was less difficult in the case of the aorta, where the vessels are largely in the outer media, but was fraught with difficulty in the case of the vena cava, where these vessels almost reach the intimal surface. Nevertheless, it can be seen from Table 4 that values up to 351 mg. of surface scrapings derived from 300 sq. cm. of aorta and 304.2 mg. derived from 300 sq. cm. of vena cava failed to yield a significant number of positive antisera. The area represented in these trials is approximately three times that of the minimal 105 sq. cm. of glomerular capillaries necessary to give a significant number of positive antisera. However, it falls short of the values for the other systemic and pulmonary capillaries. To rectify this, aorta was tested for areas of approximately 4600 sq. cm., exceeding the maximum of 2400 sq. cm. for systemic capillaries and 4100 for pulmonary capillaries. The surface scrapings from this extensive area of aorta, amounting to an average of 4.7 gm., likewise failed to yield positive antisera. It may be concluded, therefore, that the elastica interna of arterial vessels and the subendothelium of venous vessels contain little if any of the specific "nephrotoxic" antigen(s).

TABLE 4.—*The "Nephrotoxic" Antigenic Content of the Superficial Shavings from the Inner Layer of Canine Aorta and Vena Cava*

Tissue	Av. Wet. Wt. of Shavings, Mg.	Av. Surface Area from Which Shavings Derived, Sq. Cm.	Positive Antisera (Antisera Tested), No.
Aorta	180.1	150.1	0(3)
	258.3	197.5	1(6)
	346.8	300.7	0(3)
	4,496.0	4,685.4	0(3)
Vena cava	75.8	84.7	0(3)
	184.1	177.3	0(3)
	295.7	301.1	1(3)

Comment and Summary

The present experiments establish a definite association between the content of "nephrotoxic" antigen(s) in a tissue and its vascularity. In the case of large blood vessels, such as aorta and vena cava, the content of the specific antigen(s) is again related, less to anything specific in the subendothelial tissues or in the media proper than to the extent of the vascularity of the wall. The antigen(s) accordingly is in all likelihood associated with vessels of small size and more particularly with those of capillary and pre- and postcapillary dimensions. It is in these that there is a specific endothelial basement membrane which is replaced by an elastica interna in arterial vessels and which may likewise be absent in the subendothelium of veins. Capillary basement membranes, according to electron microscopic observations, are char-

acterized by a homogeneous structure with varying zones of density but with no fibrillar components. They are in essence similar to glomerular capillary basement membranes. In view of the known "nephrotoxic" antigenic content of the latter, there is compelling reason to believe that the "nephrotoxic" antigen(s) in tissues generally is a component of the capillary and pre- and postcapillary basement membranes.

The failure of Baxter and Goodman [4] to demonstrate the presence of "nephrotoxic" antigen(s) in certain vascularized tissues is no proof of its absence. In fact, other investigators have shown that brain, muscle, and liver contain the specific antigen(s) (loc. cit.). Ovary was not tested in the present experiments, but its counterpart, the testis, clearly contained the antigen. Spleen and adrenal have not been tested by others or by us, but in view of their parenchymatous and cellular nature there is reason to believe that inadequate amounts of tissue were used for immunization and that antibody production to the specific antigen(s) may have been suppressed by the large cellular component.

There are also quantitative differences in the amount of "nephrotoxic" antigen(s) in different capillary beds. However, the present findings are not in accord with those of Baxter and Goodman.[4] In order to compare the concentration of the specific antigen(s) in different tissues, the following seemed essential. It was preferable that at least all the cells of the tissue be removed in order to reduce the number of nonspecific antigens. The minimal amount of such tissue had to be determined which was necessary to produce positive "nephrotoxic" antisera under rigid standard conditions. The weight of the tissue, however, was an uncertain basis for comparison of the concentration of the specific antigen(s), since there were varied amounts of stroma and other elements, such as melanin, included in the preparation of the acellular material used for immunization. In view of the apparent presence of the antigen(s) in capillary basement membranes, the only certain basis for such a comparison was the determination of the area of capillary bed in the minimal amount of tissue that was employed. Owing to the overwhelming difficulties in determining the total volume or area of a capillary bed, the present results are based on the findings in four tissues, namely, renal glomeruli, choroid plexus, ciliary processes, and lung.

It is estimated that of these four tissues, the capillaries of renal glomeruli contain 10 to 20 times more "nephrotoxic" antigen(s) than those of chorioid plexus or ciliary processes and 40 times more than those of lung. Interestingly enough, this is a relationship in keeping with the hydrostatic or filtration pressures to which the basement membranes of these capillaries are exposed. If it is assumed that other systemic capillaries, which are exposed to a hydrostatic pressure of 25-30 mm. Hg, have an amount of "nephrotoxic" antigen(s) equivalent to that of choroid plexus or ciliary processes, then it would no longer be a question of different tissues having different amounts of the specific antigen(s). It would rather be that per unit area of capillaries, glomeruli have the highest concentration, other systemic capillaries an intermediate value, and pulmonary capillaries the least. It may be otherwise for some systemic capillaries, such as the sinusoids of spleen or liver.

It is reasonable to relate the high concentration (in terms of unit area) or probable density (in terms of unit volume or mass) of the "nephrotoxic" antigen(s) in glomerular capillary basement membranes to the production of a glomerulonephritis with heterologous antiorgan antisera. That the kidneys and in all likelihood the glomeruli rapidly clear the circulating blood of its content of specific "nephrotoxic" antibodies is well exemplified by the following experiments. Sarre and Wirtz [80] clamped the artery to one kidney of an animal and then injected a "nephrotoxic" antiserum intravenously. The clamp was removed 15 minutes after the injection. The clamped kidney subsequently failed to develop or

at least developed a mild glomerulonephritis, whereas the unclamped kidney developed classically severe lesions. Within the period of 15 minutes, therefore, the circulating blood had been so depleted of its "nephrotoxic" antibody content that the clamped kidney no longer was able to develop a full-blown glomerulonephritis. Conversely, when both renal arteries were clamped for 15 minutes, both kidneys developed glomerulonephritis. We[77] reported that if one ligated the ureter of a dog and allowed the kidney to become hydronephrotic over a 3- or 10-week period, in effect the blood flow to this kidney was so reduced that except for scattered minor lesions it failed to develop a classical glomerulonephritis when the animal was given intravenous injections of a potent rabbit anti-dog-glomerular-basement-membrane antiserum. If the antiserum was injected after the renal artery to the opposite healthy but compensatorily hypertrophied kidney had been clamped, here again, subsequently, this kidney but not the hydronephrotic one developed a classical hemorrhagic glomerulonephritis even though the clamp was retained on the artery for half an hour. In other words, once the renal circulation had been excluded, the "nephrotoxic" antibodies in the circulating blood stream were not appreciably depleted or cleared by the vast extrarenal capillary bed within a half hour at least.

It is unlikely that the differences in concentration of the specific antigen(s) in these capillary beds could completely explain these observations. Other factors undoubtedly are at play. Aside from the large volume of blood flowing through the kidney (one-third of the cardiac output in man) and the high filtration pressure within the glomeruli which would tend to bring the specific antibodies more rapidly and more easily in contact with the antigen, there are the recent electron microscopic observations that the endothelium of the glomerular capillaries is an exceedingly thin porous or fenestrated membrane permitting ready contact of antigen and antibody. In capillaries elsewhere, the endothelium is as a rule an intact well-bordered cell serving as a barrier to such union of antigen and antibody. However, according to Maynard, Schultz, and Pease,[54] a fenestrated endothelium is found in other extraglomerular capillary sites, including, according to Pease[79] the intertubular cortical capillaries of the kidney. It may be that the "nephrotoxic" antigen(s) is so bound in extraglomerular capillary basement membranes that it is not as available to unite with the specific antibody as it is in the case of the glomerulus. This, in conjunction with the lower concentration of the specific antigen and an endothelial barrier in extraglomerular capillaries, may explain the lag in their uptake of circulating specific "nephrotoxic" antibodies as well as the lack of any morphologic vascular changes.

It has been our contention that collagen forms anywhere from 76% to 83% of the total composition of glomerular capillary basement membrane,[32] despite the lack noted by electron microscopic studies of fibrils, banded or otherwise, within it. This, together with muco- or glycoproteins and a possible lipid component,[81] as indicated by chemical analyses of glomeruli, tends to bring its composition in line with that of the broad group of reticulins as defined by Robb-Smith.[40] These contain a complex glycolipoprotein and a collagenous base. It is understandable how there very well might be antigens in common within these two types of structures not necessarily related to the "nephrotoxic" one. Since extraglomerular capillary basement membranes behave in great part in the manner of glomerular capillary basement membrane, particularly in the studies with fluorescently tagged antibodies, it is not too remote to suggest that capillary basement membranes generally have a comparable composition. It is tempting, too, to speculate that in the make-up of these vascular membranes the "nephrotoxic" antigen(s) represents a distinctive and specific chemical substance which may play an important role in the

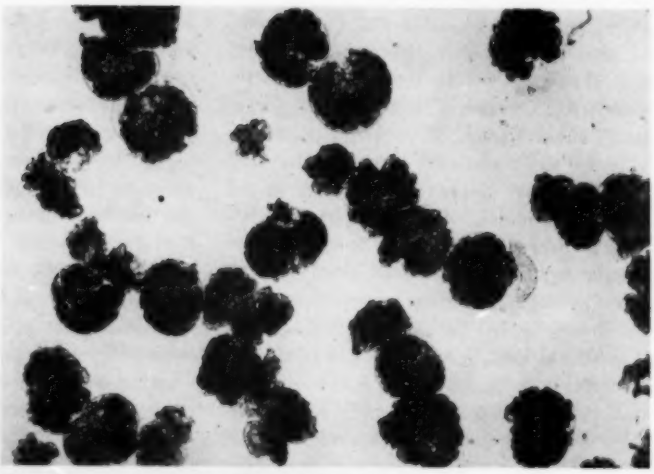

Fig. 1.—Isolated latex-filled renal glomeruli; reduced 20% from mag. × 50.

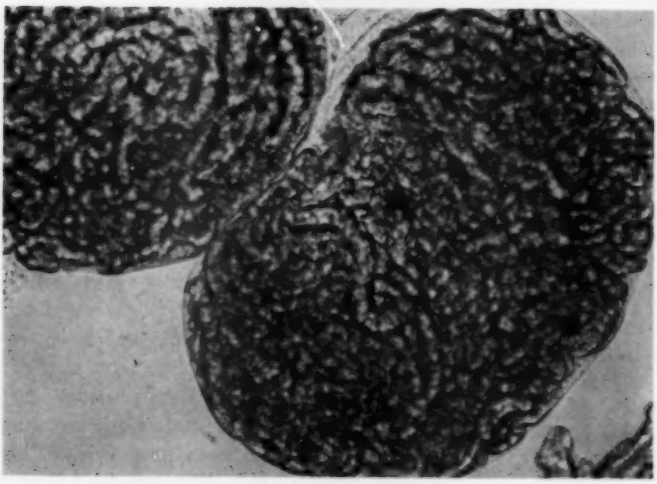

Fig. 2.—Isolated latex-filled renal glomeruli seen at higher power; reduced 20% from mag. × 350.

permeability or filtration qualities of capillaries.

Conclusions

The acellular components of avascular, poorly vascular and highly vascular canine tissues were tested for their "nephrotoxic" antigenic content. It was shown that vascularized structures only contained the specific antigen(s).

With reference to the size of the vessels concerned, it was found that the "nephrotoxic" antigenic content of large vessels was related to the abundance of fine vessels of the vasa vasorum in their medial coats rather than to any specific subendothelial or medial tissues.

Applying these findings to the facts gathered regarding the presence of the "nephrotoxic" antigen(s) in glomerular capillary basement membrane, the conclusion was reached that this specific antigen(s) was in all likelihood to be found within and was part of capillary and pre- and post-capillary basement membranes universally. The lamina of the elastica interna of the arterial system and probably the fine elastic fibers of the veins in serving as a substitute for a capillary basement membrane contained little if any of the specific antigen(s).

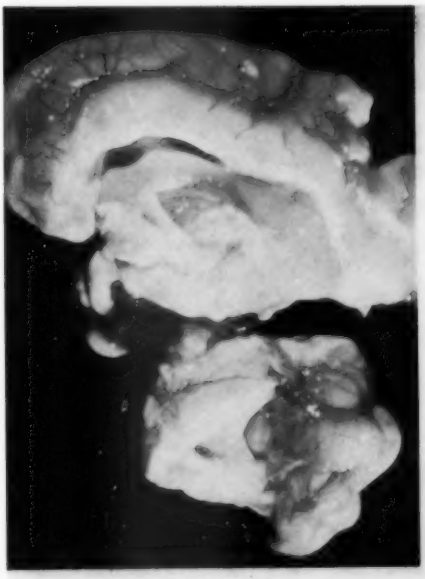

Figure 3

Fig. 3.—Brain, to show latex-filled choroid plexuses of lateral and fourth ventricles.

Fig. 4.—Enlarged view of the latex-filled choroid plexus from the lateral ventricle, to show tortuous close-set capillaries. Larger vessels, as seen to either side of the photograph, were dissected away; × 18.

Fig. 5.—The inverted eye submerged in isotonic saline solution, to show the yellow latex-filled ciliary processes.

Fig. 6.—Enlarged view of isolated latex-filled ciliary processes. Note the rich close-set capillary bed; × 13.

It was further shown that the concentration of the "nephrotoxic" antigen(s) in glomerular capillary basement membranes was 10 to 20 times greater than those in extraglomerular systemic capillaries such as in chorioid plexus and ciliary processes and 40 times greater than those in pulmonary capillaries. These differences could be related to the tensions placed upon, and the filtration or hydrostatic pressures applied to, the capillary basement membranes in these different locations. They seemed to serve as an index of the structural and functional adjustments of the capillary basement membranes to different hemodynamic factors. In that regard, it was suggested that the "nephrotoxic" antigen(s) may be a specific chemical substance capable of playing an important role in the permeability or filtration qualities of capillaries.

The high concentration of the "nephrotoxic" antigen(s) in glomerular capillary basement membranes appeared to be an important but not the sole factor in rapidly clearing the blood stream of injected "nephrotoxic" antibodies and in accounting for the ensuing glomerulonephritis. Conversely, the lower concentration of the specific antigen(s), an endothelial barrier and a possible bonding of the antigen(s) so that it was not as readily available to circulating "nephrotoxic" antibodies, might explain the lag in the uptake of these antibodies and the failure in the production of any morphologic changes in extraglomerular capillaries.

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Vascular Changes in the Arthus Phenomenon

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The tissue reactions that characterize anaphylactic shock likely are not due, as believed in the past, to "free" antibodies, to the toxicity of the "circulating" antigen-antibody complex, or to a substance derived from an antigen-antibody interaction either in the blood stream or in the milieu intérieur. Rather, anaphylactic shock probably involves disturbances in or on susceptible cells, such as histiocytes and smooth-muscle cells, caused by a primary antigen-antibody coupling. This "cellular" hypothesis, supported by numerous immunologic experiments and more recently by the work of Shields Warren and F. J. Dixon,[1] is decisively substantiated by the well-known Schultz-Dale experiment: The involuntary muscle (ileal strip, uterine horn) contracts in vitro, in a Locke solution bath, upon addition of minimal traces of the specific antigen. The contraction takes place even after the sensitized uterus has been perfused with isotonic solution prior to its removal from the body. Similar reactions occur also in vivo: Intermittent segmental arteriolar spasm can be visualized in sensitized rabbits carrying a transparent Clark chamber grafted in their ears when a shocking dose of antigen is instilled into the chamber.[2]

The present investigation is concerned with lesions of the smooth-muscle fibers during anaphylaxis. Effector organs, such as the bronchioles of guinea pigs dying in anaphylactic shock, were not believed to be suitable, because in this situation death due to respiratory obstruction and suffocation is almost instantaneous, leaving no time for the development of lesions of the bronchiolar muscles. I therefore selected for study the muscle fibers of the blood vessels in the focal anaphylactic reaction of the Arthus type, in which repeated local administration of antigen could exert a cumulative effect upon the morphology of the muscle coats.

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Material and Techniques

The classical Arthus experiment was duplicated.[3] Rabbits were used; normal horse serum served as antigen; injections were made subcutaneously in the abdominal wall at six- to seven-day intervals in amounts of 5 ml. Injections were continued until local necrosis replaced the indurated swelling of the skin (seven weeks). In a few rabbits necrosis failed to appear even after 10 weekly injections. One day prior to and one day after each injection one or two rabbits were killed by a blow on the head and tissue specimens from the abdominal wall were prepared. In some instances, additional animals were killed in midweeks.

To minimize artifacts due to oozing out of the tremendous edema and to the great mobility of the various tissue layers over each other, a cork frame almost the size of the rabbit's abdomen was used. The borders of this cork frame had been pierced by pins which were pushed as deep as possible into the rabbit's skin. The latter was dissected along the outside borders of the cork frame and secured to it with additional pins. The preparation comprised skin, subcutaneous tissue, muscle planes, and parietal peritoneum and contained the focal zone and its periphery up to unaffected areas. The specimen was immediately placed in a refrigerator, from which it was removed only when it developed a rubbery consistency; it was then cut with a razor blade into small fragments, their location of origin being carefully identified.

Several chromate fixing fluids were used (Zenker, Helly, Maximow, or modifications thereof). Paraffin blocks were sectioned either perpendicular to the abdominal wall (sagittal and coronal sections) or tangentially to it (frontal sections). The latter furnish more information, especially at certain levels; the former are of topograpical value. Masson's trichcrome stain was used routinely. Other stains used on alternate sections, when warranted, included Mallory's or Heidenhain's iron hematoxylin, Nocht-Maximow's azure-eosin, Mal-

lory's phosphotungstic acid hematoxylin, aldehyde fuchsin, Gomori's or Laidlaw's silver impregnation for reticulin, and Weigert's fibrin stain.

Results

The lesions in general did not differ from those previously described by Rössle [4,5] and his school [6,7] and can be termed a "quantitatively" enhanced inflammation. The swelling of the connective tissue bundles was enormous and caused a disappearance of the fibroblastic nuclei and compression of the capillaries, so-called focal blanching. The edema was overwhelming and was not resorbed even toward the end of the experiment. Blood stagnation, fibrin deposition, fibrinoid degeneration, and accumulation of polymorphonuclear cells, and especially eosinophils, were more conspicuous than in normergic inflammations. Walling off of the necrotized area occurred earlier and was more complete. Scarring was also hastened. The entire evolution was, to use an expression coined by Rössle, "stormy."

Perivascular histiocytic granulomata were present. In previous investigations [8] this finding, in guinea pigs, differentiated "qualitatively" the hyperergic inflammation from a normergic one. The presence of these granulomata in another species substantiates their importance as characteristic of hyperergic inflammation.

This granuloma failed to appear, in the rabbit, prior to the third or fourth weekly injection, whereas in the guinea pig the granuloma was present a few hours after the shocking injection. Moreover, these granulomata were, in the rabbit, scarce, inconspicuous, and best described as miniature granulomata, whereas in the guinea pig they constituted the most important feature. The diffuse accumulation of histiocytes and related cells was more impressive in the rabbit than in the guinea pig. It may be spoken of as a generalized histiocytosis. Plasma cells were fewer in the rabbit than in the guinea pig; eosinophils, slightly more numerous. All other signs, such as edema, accumulation of polymorphonuclear cells, and fibrinoid degeneration, were almost identical in both species. A fibrotic metamorphosis of the granuloma, as seen in the guinea pig, was not observed in rabbits, perhaps because each new weekly injection induced new granulomata and prevented the older ones from undergoing fibrotic changes.

In addition to the lesions noted above, vascular changes were observed which have not been seen in the guinea pig and are not described in the literature.

Vascular Changes.—The changes in the vessels ran the entire gamut from disappearance of the endothelial cells (Fig. 1) to the disintegration of the vessels as a whole. Further changes, described below,

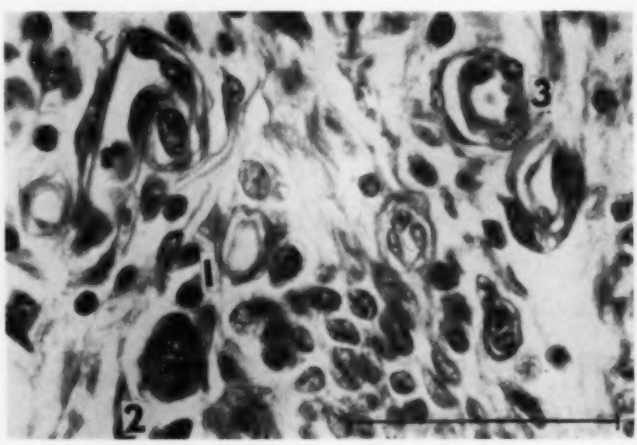

Fig. 1.—Group of vessels: *1*, vessel without endothelial nuclei; *2*, slit lumen, heavy muscle coat; *3*, histiocytes outside the media forming a reinforcing ring. Four weekly horse serum injections. Helly, Masson; scale, 50μ.

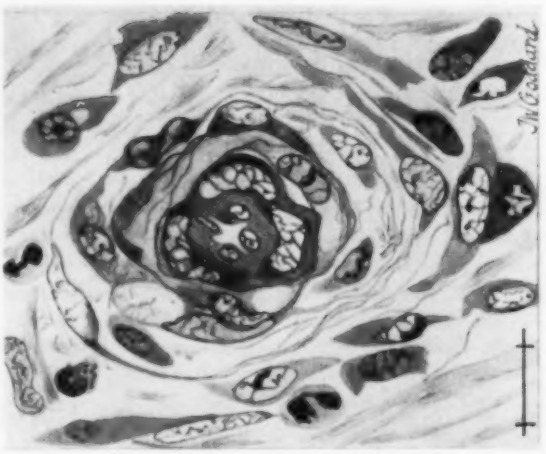

Fig. 2.—Arteriole. The clamping contraction has brought about narrowing of the lumen and changes in the shape of the endothelial cells. The original media is made up of a ring of three dark cells with hypertrophic nuclei. This coat is reinforced by two apposed rings, made up of histiocytes. Some of the latter are anastomosed and may or may not simulate smooth-muscle fibers. The significance of their vacuoles is not clear. Even outside the apposed rings the histiocytes and their derivative cells are circumferentially arranged. Rare plasma cells, polymorphonuclear cells, and eosinophils. Seven weekly injections. Zenker, Masson; Camera lucida drawing. Scale, 10μ.

appeared to be of particular interest because they can be correlated with the physiology peculiar to the hyperergic inflammation and because they manifested in the arteriolar tree an "allomorphism" not evidenced in normergic inflammatory reactions.

Arterioles.—In the granulation tissue or in its rim bordering the central necrotic mass arterioles showed markedly narrowed lumina and thickened media (Figs. 2, 3, 5, and 6), the latter representing either hypertrophy, hyperplasia, or both. In small arterioles the number and volume of nuclei were increased beyond normal ratios (Fig. 6). Participation of greater numbers of contractile elements than are normally possessed by the media was demonstrable. These contractile cells originated either in the media (Figs. 6, 7, and 12) and at the expense of muscular elements proper or, in adventitia, from histiocytes (Figs. 2, 3, 5, 7, and 8).

In Figure 8 rings of smooth-muscle fibers are shown both inside and outside the adventitia. One ring appeared to be derived from the media proper, spreading peripherally into the adventitia, where it entered into contact with neighboring muscle fibers. The

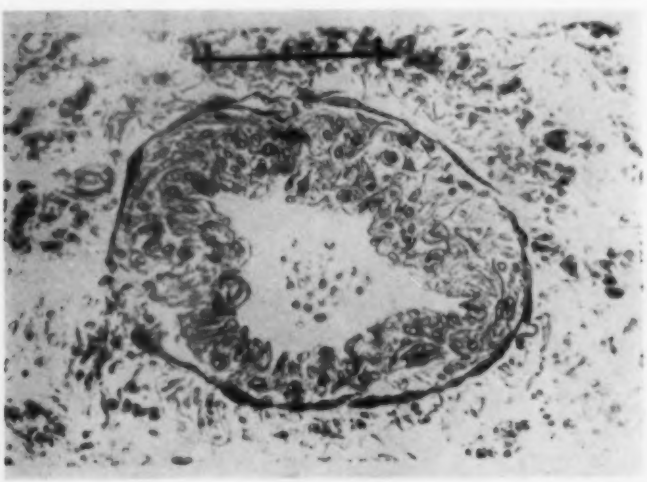

Fig. 3.—Artery. Thickened media, with numerical increase of nuclei and whorling of smooth-muscle fibers. Adventitial ring of myoblasts. Six weekly injections. Helly, Masson; scale, 100μ.

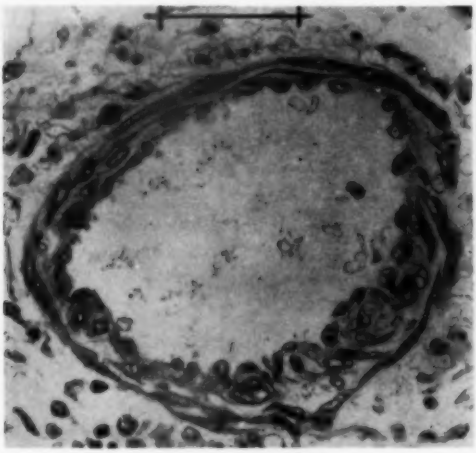

Fig. 4.—Arteriole. Circular smooth-muscle fibers interspersed with few whorled or longitudinally running elements. Moulded on the media and separated from the latter by connective tissue fibrils is a ring of newly formed contractile cells. Three weekly injections. Helly, Masson; scale, 50μ.

periadventitial ring was broken in parts. Figure 4 is similar to that just described, except for the tendency of the smooth-muscle fibers to become free and to spread inwardly and outwardly. In the example of Figure 3 serial sections revealed the external ring to be circumferential, but in some cases the periadventitial fibers ran in spiral fashion.

In Figure 5 smooth-muscle fibers are demonstrated which reinforced an arteriole on one side only; some are cut obliquely, and some, longitudinally, although the media was almost perfectly transversely sectioned. These bundles were not in immediate contact with the media but were separated from it by intervening argyrophilic fibers. The same Figure shows histiocytes in apparent transformation into contractile elements. A few of these latter cells showed occasional myofibrils. Those without myofibrils had rather spindly cell bodies and tapered ends that resembled myoblastic precursors (Figs. 2 and 8). The newly formed contractile cells, commonly seen around arterioles, were in rare instances also found in the media and subintima (Fig. 10). Here they ran at right angle to the medial coat.

Certain morphologic changes in the nuclei were observed. Some of them were twisted, showing indentations that suggested a spiral torsion (Fig. 6). Twisted nuclei of a simi-

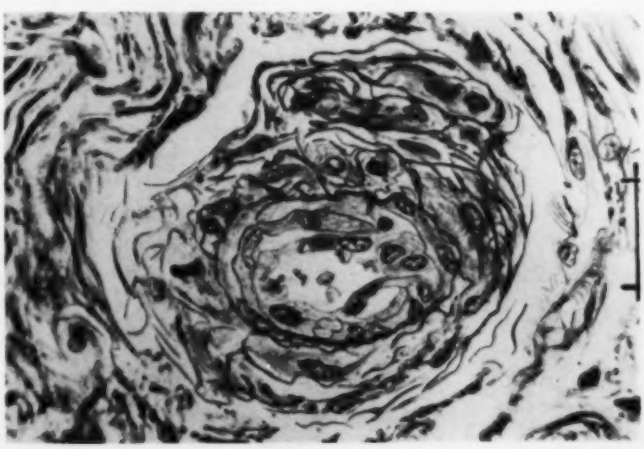

Fig. 5.—Arteriole. Desquamating intima. Some of the medial smooth-muscle fibers run almost perpendicularly to the circular medial coat. Most peripherally, and circumscribed by argyrophilic fibrils, is an ectopic bundle of smooth-muscle fibers. Seven weekly injections. Gomori's silver impregnation and Masson's trichrome; scale, 30μ.

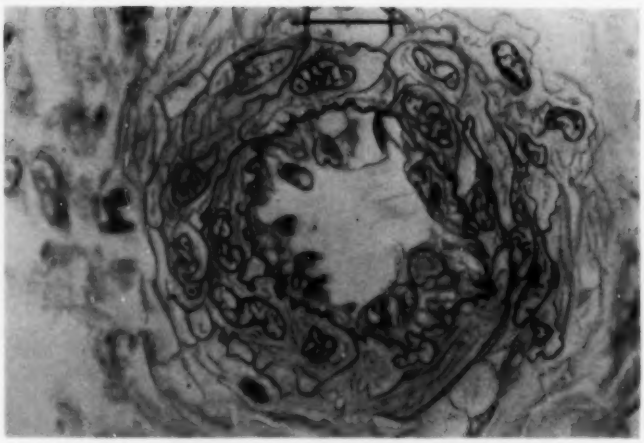

Fig. 6.—Arteriole with twisted medial nuclei, thickened media. Delicate elastic fibrils circumscribe almost every single smooth-muscle fiber. Seven weekly injections. Helly; aldehyde fuchsin and Mallory's iron chloride hematoxylin; scale, 10μ.

lar appearance, incidentally, are not uncommon in the smooth muscle of invertebrates.

In Figure 11, the lumen is almost entirely obstructed by an ingrowth of medial fibers accompanied by connective tissue bundles. In this plug the shadowy and isolated smooth-muscle fibers still showed evidence of contraction.

Elastic fibers underwent practically no changes. More rarely, an arteriole of the essentially muscular type was observed to contain delicate elastic fibrils rather ectopically (Fig. 6). A complete periarteriolar ring was the exception.

The findings described above were interpreted as reflecting functional changes; they were not lesional but were rather adaptative in character. One morphological lesion was observed, however. This was an edema which dissected between and isolated the arteriolar smooth-muscle fibers (Fig. 12). It was part of the general edema which pervaded the focal area and stretched outwardly. Despite the arteriolar edema and the consequent separation of the individual fibers, the muscle was found to be contracted at the moment of fixation.

It should be emphasized that the above findings were present in or around the granulation tissue and absent in the arteriolar tree of the less involved dermis and muscle wall. That the changes were seen only in the arterioles of the granulation tissue, and not elsewhere, suggests that these

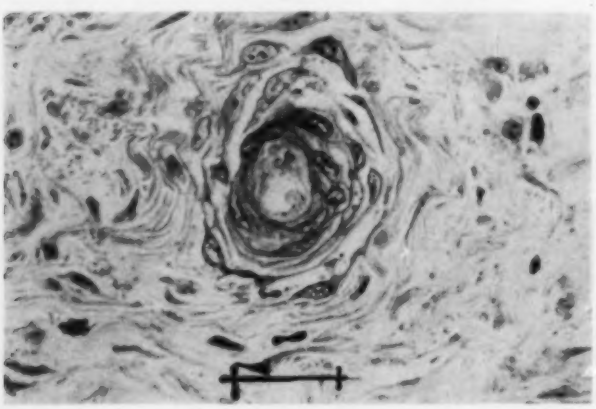

Fig. 7.—Arteriole with hyperplasia and hypertrophy of media and histiocytes moulded upon the circumference of the vessel. Their spindled shape and huge nuclei are more reminiscent of myoblasts than of histiocytes. Eight weekly injections. Helly; Mallory's iron chloride hematoxylin; scale, 30μ.

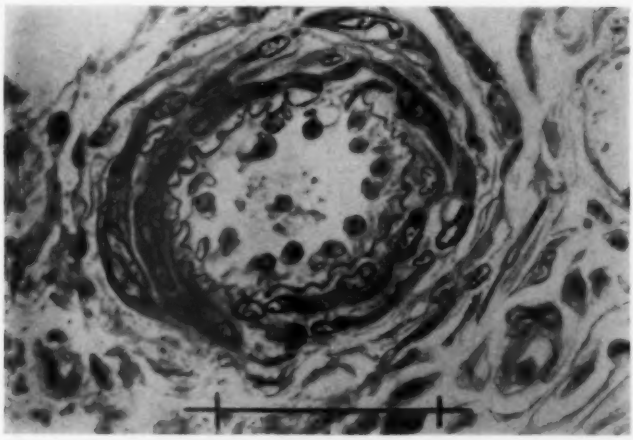

Fig. 8.—Arteriole with media surrounded by apposed smooth-muscle fibers. Outside the adventitia are a few aberrant smooth-muscle fibers. Four weekly injections. Zenker; Mallory's phosphotungstic acid hematoxylin; scale, 50μ.

arterioles were recently formed through an arteriolization of the new capillaries contained in the granulation tissue.

Venules.—In most venules the endothelium was detached from its moorings. In many instances endothelial cells were found free in the lumen. Whether still anchored or not, the endothelial cells showed an increase in size and number and a rounding of their nuclei. The last sign perhaps forecast an impending monocytoid metamorphosis. With more advanced endothelial hyperplasia, the increased cells conglomerated at one point, in a protruding tongue-like fashion, the angular extremity of which penetrated into the lumen (Fig. 13).

Endothelial spurs or slings also penetrated into otherwise unblocked and perfectly patent and nonthrombosed venous lumina (Fig. 14). Here they became elongated and tended to anastomose, forming septa (Fig. 15). Endothelial cells covered the surfaces of the ingrowths with only a scant interposed ground substance that contained occasional broken, coiled, or wavy elastic fibrils. In sections impregnated with silver the stroma was made up almost entirely by argyrophilic fibrils. These were continuous or interrupted, wavy or interlaced in fine meshes, or simply granular. The reticulin skeleton was continuous with the reticulin scaffolding of the vein.

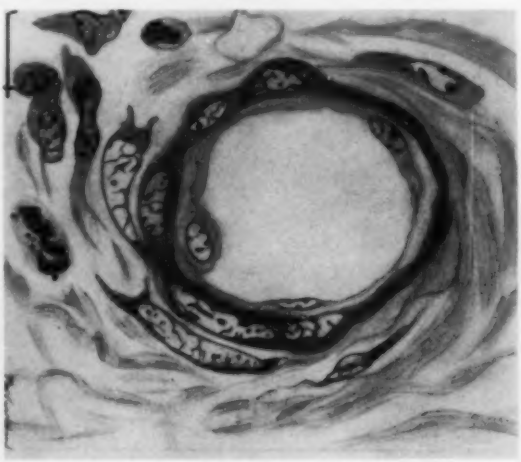

Fig. 9.—Arteriole with large lumen and irregular medial contour. Circumscribing the latter are a few histiocytes, with wavy protoplasmic ends, not yet anastomosed. Seven weekly injections. Zenker; Mallory's iron chloride hematoxylin; Camera lucida drawing. Scale, 10μ.

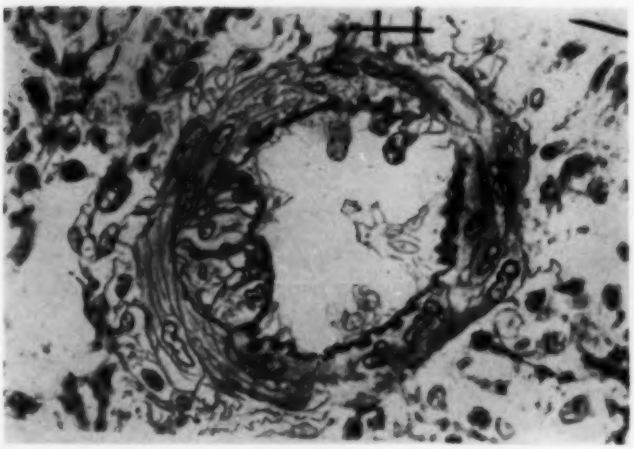

Fig. 10.—Arteriole with an ectopic subintimal patch of smooth-muscle fibers which run perpendicularly to the media. Each muscle fiber in the patch is wrapped by elastic fibrils. Six weekly injections. Zenker; aldehyde fuchsin; Masson; scale, 10μ.

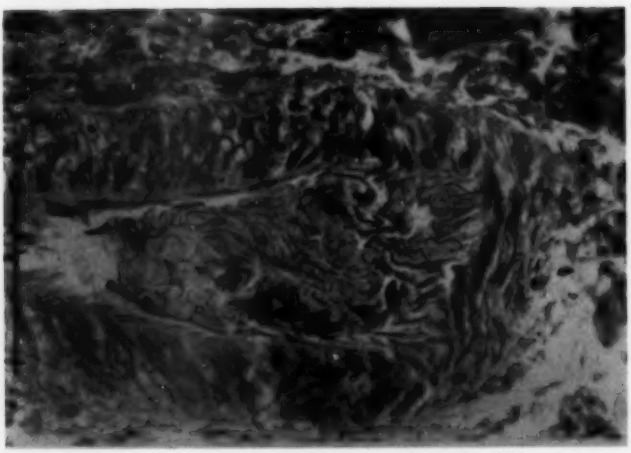

Fig. 11.—Artery with intraluminal plug of connective tissue fibers, interspersed with few still contractile shadows of smooth-muscle fibers. Seven weekly horse serum injections. Zenker; Mallory's phosphotungstic acid hematoxylin; scale, 100μ.

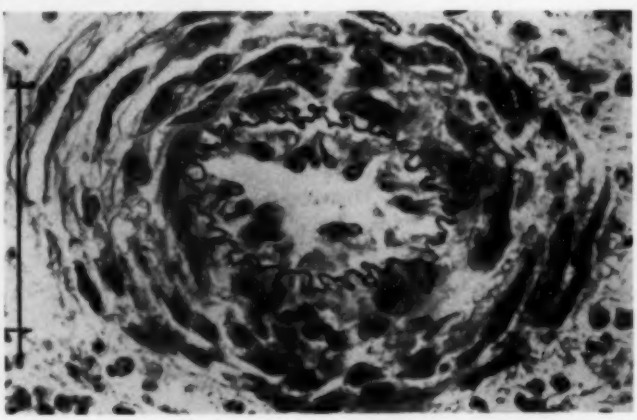

Fig. 12.—Arteriole with edema of media. Though dissociated, the smooth-muscle fibers show evidence of contractility. Two weekly injections. Zenker, Masson; scale, 100μ.

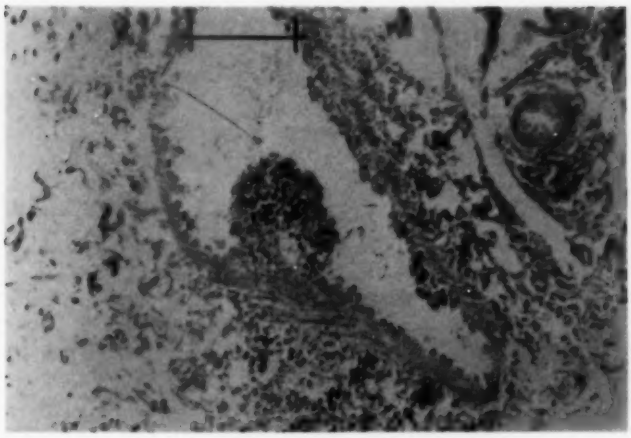

Fig. 13.—Vein containing an intraluminally protruding hillock of histiocytes. Lumen is otherwise patent. Five weekly injections. Helly, Nocht-Maximow; scale, 100μ.

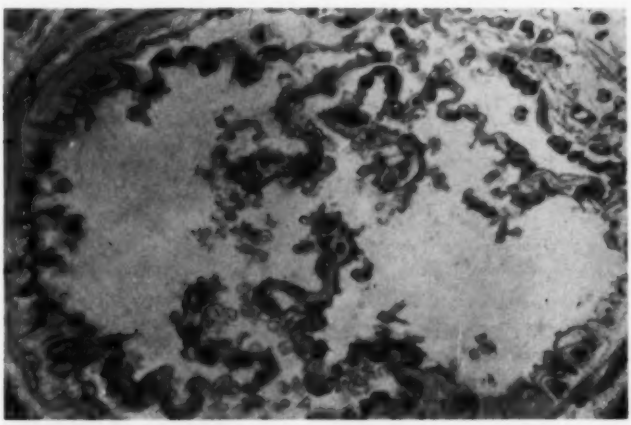

Fig. 14.—Vein with sling-like formations penetrating into the patent lumen. The endothelial lining on both surfaces is visible. The ends of the digitations are not yet anastomosed. Four weekly injections. Helly, Nocht-Maximow; scale, 50μ.

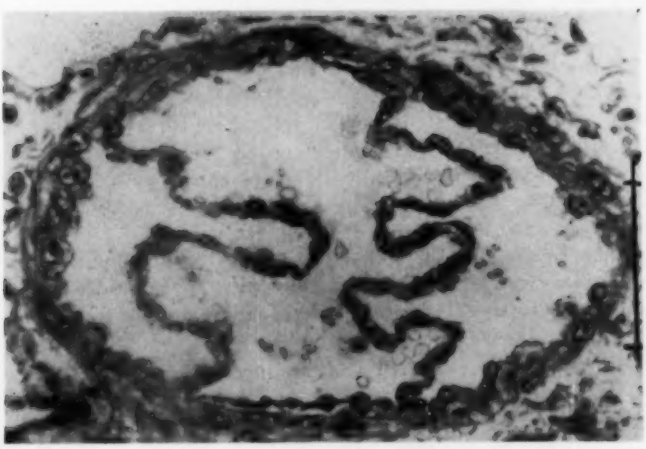

Fig. 15.—Vein with anastomosed sling-like septa dividing the lumen into three compartments. Five weekly injections. Zenker, Masson; scale, 50μ

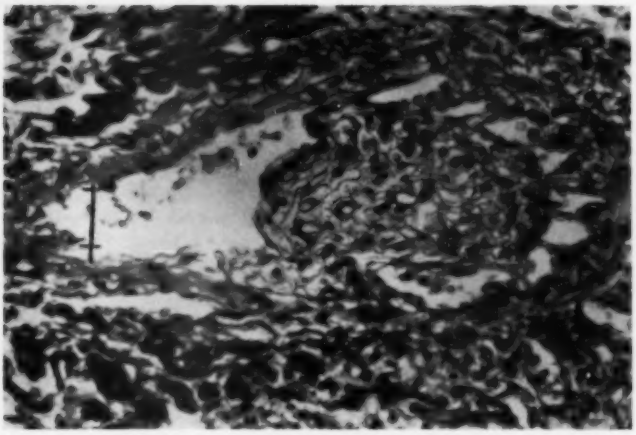

Fig. 16.—Vein whose short septa terminate upon a formation made up of anastomosed histiocytes (reticulum cells). This miniature intraluminal lymph node-like structure is pervaded by capillaries. Seven weekly injections. Zenker, Mallory's phosphotungstic acid hematoxylin; scale, 50μ.

In some cases the ingrowing digitations came into contact and engendered a central or eccentric mass. This was covered by endothelial cells and contained a core of histiocytes. The latter were arranged in a reticulum-cell pattern and resembled a miniature lymph node, without its lymphocytic population. The core contained a few capillaries (Fig. 16).

A physiological interpretation of the intraluminal digitations seems difficult. It is ventured that owing to their elasticity they may create venous eddies. This turbulence in the fluid, coupled with a slow flow rate, could increase the lateral pressure and hence keep the vein, which is bathed by edema, from collapsing. They may also act as valves or as internal sphincters and as such regulate the pressure in the capillaries.

The venous media was involved only in exceptional instances, in contradistinction to the findings in the arterioles. Lesions of the media suggested impending destruction of the vessel. In such vessels the wall of the venule showed marked thinning out in one or more areas of the cross section, alternating with irregular sausage-like thickening in other areas. There were few nuclei and marked fuchsinophilia, the fuchsin component being taken up even when applied simultaneously with other dyes (Xylidine-ponceau, or Biebrich scarlet and orange G; modified Masson's trichrome). In such cases the argyrophilic reticulum presented a coarse, clumped, and broken irregular meshwork.

The veins that showed the most marked endothelial hyperplasia and septation were located marginally, mostly in or next to the granulation tissue that walled off the residual edema or the central necrotic mass. Probably the involved veins were newly formed and originated from the capillary network of the granulation tissue.

Summary

The local subcutaneous anaphylactic shock, the Arthus phenomenon, when studied in the rabbit showed the following.

The presence of miniature histiocytic perivascular granulomata. These granulomata were less striking than in the guinea pig, where they constituted the dominant and distinctive lesion. Granulomata add a "qualitative" character to the hitherto "quantitative" change of an hyperergic inflammation. The granuloma appeared slowly, not before the third or fourth weekly injection of specific antigen.

Invasion by histiocytes, and by cells derived from histiocytes, was marked, and diffuse histiocytosis was more extreme than in the guinea pig.

Arterioles and venules showed characteristic changes, which were totally absent in the guinea pig. They consisted of hyper-

plasia and hypertrophy of the muscle coat in the arterial branches, coupled with the presence of surrounding rings of myoblasts or histiocytes in myoblastic metamorphosis, as well as development of aberrant muscle fibers in the media. In the venules there was an endothelial ingrowth leading to valve-like structures and to formation of intraluminal septa.

A normergic inflammation during its evolution exerts an impact only upon true mobile or mobilizable mesenchymal cells. An hyperergic inflammation, at least in the rabbit, induced, in addition to its effects upon germane mesenchymal cells, changes in mesenchymatous derivatives, the smooth-muscle cells of the media.

The repair went beyond normal limits in the rabbit and engendered aberrant structures (allomorphism), whereas during the repair of the hyperergic response in the guinea pig the arteriolar tree reduplicated normal structures (isomorphism). This may represent a morphologic adaptation to the functional changes occurring in local shock, possibly to a spastic constriction in the arterial twigs. The functional significance of the venous septa was not clear.

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Time-Intensity Factors in Radiation Response

II. Some Genetic Factors in Brain Damage

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In a previous study [1] it was shown that, in several strains of mice and in rats, large doses of radiation given in very short periods of time could produce severe damage to the central nervous system or even be immediately lethal. Extremely intense radiation (30,000 rep of 2.5 mev electrons in one second, for example) could kill a mouse "instantly," that is, the animal became convulsive and was dead within seconds after the irradiation. The crucial part of the brain that seemed to be involved in this reaction was the lower half of the medulla, for shielding this part prevented immediate death. Animals that survived such doses developed destructive lesions of the forebrain, the retina, and sometimes the vestibular and internal ear apparatus, with severe accompanying disorders of function and convulsive episodes. In these studies, special attention was not given to genetic factors. In another study,[2] the response of four genotypes to certain types of brain injury and convulsions was compared. It was shown that methionine sulfoximine, an antimetabolite of glutamine and methionine that produces epilepsy-like convulsions in a number of mammals, would severely damage the forebrain of DBA/2 mice. It produced scant damage in some other strains of mice and in rats but failed conspicuously to produce lesions in the C57 Black 6 mice, although somewhat paradoxically they were susceptible to the lethal effects of the seizures. DBA mice when compared with C57 mice have inborn errors of brain metabolism characterized by deficiencies of certain enzymes concerned with oxidative phosphorylations,[3] and this may be related to their susceptibility to seizures induced by a number of agents and especially loud noises ("audiogenic seizures").[4] The susceptibility to forebrain damage by methionine sulfoximine may be partly explained by assuming that in an animal whose energy yielding processes for brain metabolism are already subnormal interference with the metabolism of glutamine, a prime metabolite of the brain, would have severe consequences.

These studies, in which the patterns of convulsions, liability to death, and production of brain lesions were strongly characteristic for the genotype, suggested that the same sort of study ought to be made comparing effects of radiation on the brains of mice of several genotypes. Experiments were carried out in which the acute effects of large doses of high-intensity radiation on the brains of four strains of mice, DBA/2, DBA/1, LAF_1 and C57 Black 6 were compared. The DBA's and LAF_1's were frequently killed immediately, but the C57's were not, and there were differences in the pathologic lesions as well as similarities between strains. However, the mechanisms involved were complex and not susceptible to a simple explanation. This report will tell about the experiments and comment on some of the factors that may govern the responses in the brain following irradiation.

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Experiments

One hundred male and female mice, twenty-five of each of the four strains mentioned, 3 to 5 months old and recently obtained from the Roscoe B. Jackson Memorial Laboratory in Bar Harbor, Maine, were exposed to 30,000 rad of 3 mev electrons in one second. The DBA and C57 strains were chosen because data on their brain metabolism[8] and their physiologic responses to convulsants[4,5] have been gathered and we are presently comparing their responses to several metabolic inhibitors including the methionine sulfoximine already noted.[2] The LAF_1 mice were included because they are first-generation hybrids and they have become popular in radiation experimental work. The method of exposure has been given in detail before,[1,6] but, in summary, it consisted of submitting the animals to the radiation by carrying them through the center of the electron beam in cardboard boxes on a conveyor belt. Eight animals were exposed at a time, two from each strain. The pathway through the beam was arranged so that the transverse distribution of the electrons was uniform and a calculable time-integrated dose along the animal was uniform. The dose down through the animal was not uniform: the ionization in depth curve[3,6] for 2.5 mev electrons in materials of low atomic weight, from which doses are estimated, is such that close to the surface ionization is about 60% of maximum, then it rises to 100% at about 0.4 cm. and falls to 60% at a depth of 0.9 cm. There is virtually no ionization at a depth of 1.4 cm. For 3 mev electrons the depth of penetration is slightly greater, but the maximum dose curve is for practical purposes similar. In previous experiments the dose was expressed in rep, but in the present ones the rad was used (30,000 rad is approximately equal to 36,000 rep. In previous experiments the rep was a unit taken to be the absorption, on the average, of 83 ergs per gram. The rad unit, as used in these experiments, is defined as an energy absorption of 100 ergs per gram.) The dose reported in these and previous experiments[6,7] was the average shown in the reference curve, which is 85% of the maximum. It was further estimated that the brains of the mice lay in that part of the curve where the ionization range is between 75% and 100% of maximum.

Thirty-one of the one hundred animals survived 24 or 48 hours to be autopsied (with one exception), seven each of DBA/1, DBA/2, and LAF_1 strains and ten C57's. (The exception was a female DBA/1 in late pregnancy autopsied four hours after irradiation.) Complete gross autopsies were performed, but only microscopic studies of the brain were carried out for this study. Complete serial sections were made of the brain stem and cerebellum from the midbrain to the lower end of the medulla, and multiple ribbon sections were made through the rest of the brain. These were prepared by fixation in Bouin's fluid, embedding in paraffin, and staining with hematoxylin and eosin. No animals that died during the 24 hours were autopsied because the histologic findings would have been unreliable for this kind of a study.

The animals were kept under observation during the postirradiation period, and at intervals their appearance, gross behavior, and deaths were recorded.

Results

The general quality of the behavior and physiologic responses and the essential nature of the pathologic changes following irradiation in these experiments was similar to that in previous experiments—that is, instant death, the development of severe neurologic disturbances and convulsions, and the necrosis of neural elements were not, with certain exceptions, different. The time sequences of the behavioral and physiologic reactions and the distribution of the lesions in the several strains were different, and these will be particularly dwelt on here.

Pathologic Changes.—In previous studies, when large doses of radiation were given to a variety of mice and rats certain acute changes in the brain tended to occur. After several thousand roentgens of conventional x-rays, scattered necrotic oligodendroglia resulted, the threshold for this in a number of strains of mice and rats being around 1500 r. In the same range, destruction of some granule neurons in the olfactory lobe was a regular occurrence, while neurons in the pyriform cortex and neocortex were only occasionally destroyed. The granule cells of the cerebellum were virtually never necrotized by conventional x-rays, given at about 100 r per minute up to 10,000 r, but when the rate was boosted to 1000 r per minute, or the radiation was given as megavolt electrons (cathode rays) in thousands of rep per second, cerebellar damage became conspicuous. As 10,000 r or rep was exceeded, irreversible damage to the granule cells became an increasingly regular finding, the affected cells disinte-

grating completely if the animal could be kept alive for several days. Purkinje cells and constituents of the molecular zone were less often affected. Both x-rays and megavolt electrons, given in massive doses (20,000 or 30,000 r or rep) at these very rapid rates also damaged neurons in the neocortex, hippocampus, and other areas, sometimes extremely extensively. At the same time those kinds of cells affected at the lower doses became increasingly involved as the dose was raised. Rarely these exposures also produced acute disintegration of patches of white matter—necrosis of the myelinated fibers and the associated oligodendroglia. Cell destruction occurred in the diencephalon and lower brain stem, but it was usually scant and not a prominent feature unless the dose was very high. The neurons in the reticular formation, olivary region, and dorsal cochlear nucleus of the medulla were sometimes destroyed in numbers. Meningeal reaction was absent, and morphologically evident vascular damage did not occur except for dilatation of some vessels. In young adult mice and rats up to a few months of age, embryonal neural cells persist in the forebrain beneath the ependyma of the lateral ventricles, and these were necrotized by as little as 100 to 200 r.

These generalizations are based on experiments with a variety of animals in which "common" mammalian responses were being sought, not differences. In the present experiments in which the genotype of the mice and the dose of radiation was set, many of the expected responses materialized but the distribution of lesions showed some peculiarities and there were real differences in patterns of damage between strains. Cerebellar granule-cell damage was frequent, and severe damage to neocortical neurons was seen in certain animals. Severe relatively focal damage to the lateral medulla, a pattern of damage not seen in previous experiments, and effects on oligodendroglia and some neurons in the thalamus were added features. Since a description of individual cell changes is already a matter of record,[1,7] the patterns of injury may be given here and some highlights are summarized in the Table. (Numbers of animals will be given only where the animals are referred to in more than one place in the text.) C57 mice frequently had patches (like that shown in Fig. 1), or fairly extensive regions of necrosis, of cerebellar granule cells, sometimes associated with some regional swelling of Purkinje cells and the molecular zone matrix. This chiefly affected the central part of the cerebellum, from the nodule forming the roof of the fourth ventricle upward. In some instances more lateral folia were affected. The severe cerebellar lesions usually showed a considerable degree of sharp demarcation from adjacent undamaged regions. There was also

Summary of Significant Differences and Possible Trends in Response to Irradiation

Strain	No. Autopsied	Lesions				
		Severe Cerebellar Damage	Damage of Neocortex	Necrosis of Lateral Medulla	Damage to Pyriform Cortex	Immediate Death †
C57	10	++++	++	++++		
		++++		+		+
DBA/1	7	++	++++	++++	++++	++++
			+++	+++	+++	++++
DBA/2	7	++	++++		++++	++++
			+++		+++	++++
						++++
						++++
						+
LAF[1]	7	++++	++++		++++	++++
		+++	+++	+	+++	+++

* Each + represents 1 animal.
† In 25 animals of strain.

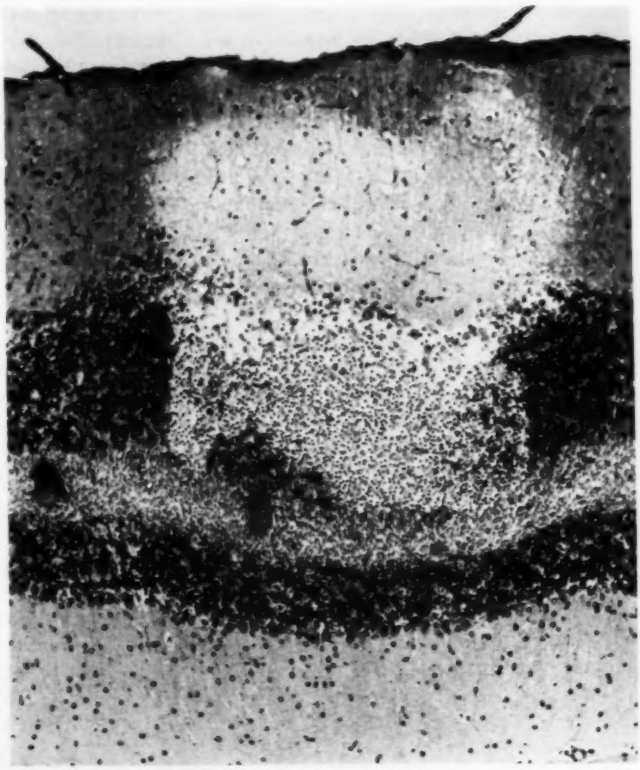

Fig. 1.—A sharply demarcated area of necrosis of the cerebellum, chiefly involving the granule cell layer. Local veins are dilated. DBA/1 mouse (No. 5). Hematoxylin and eosin; × 125.

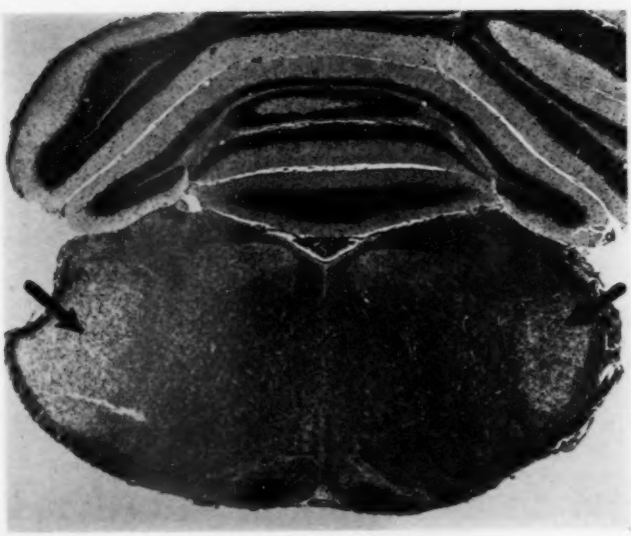

Fig. 2.—Bilateral areas of necrosis in the medulla in a DBA/1 mouse (No. 23). Hematoxylin and eosin; low power, × 125.

necrosis of granule cells in the olfactory lobes and an oval patch of necrosis in sections of the lateral medulla (like that shown in Fig. 2), involving most or all neural elements in the region. The medulla lesion was fairly demarcated and was approximately at the level between the cochlear nucleus and 7th nerve above and 10th nerve nuclei below. It was principally centered in the spinal nucleus and tract of the trigeminal and involved the restiform body, more or less, in its lower extent. Two C57 animals (No. 1 and No. 30) had extensive circumscribed regions of necrosis of the superior and inferior colliculi of the midbrain, and one of these also had necrosis of the lateral and medial nuclei of the mammillary body of the hypothalamus.

The most striking finding, however, was the virtual absence of destructive changes elsewhere in the brain of C57's (except the olfactory lobes). In only two animals (No. 1 and No. 30) was there some scattered necrotic oligodendroglia in the anterior dorsal thalamic nuclei, and in one of these there were some similar cells in the striatum and cortex. Even pyknosis (compaction) of oligodendroglia nuclei, a usually regular finding in heavily irradiated mammals, was virtually absent or not at all prominent in the eight other C57's. One can almost invariably find a few necrotic oligodendroglia and sometimes dead neurons in the pyriform cortex after heavy irradiation, but none appeared in any C57's. Altogether they differed significantly in these respects from the other three strains.

The other three groups of mice were as a whole more seriously damaged in a number of regions, and the LAF_1's were generally the worst off so far as variety and extent of lesions. There were degrees of

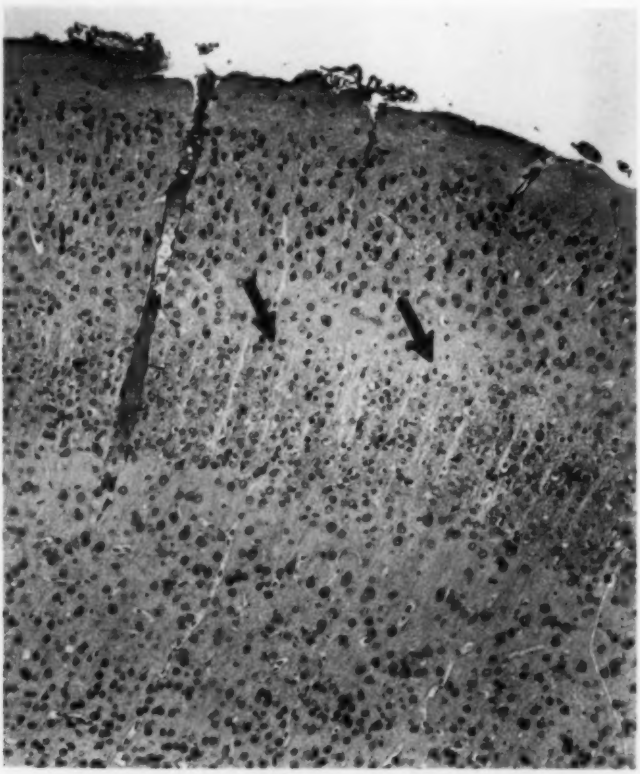

Fig. 3.—Laminar necrosis of the neocortex in an LAF_1 mouse (No. 16). Hematoxylin and eosin; ×125.

difference in the patterns of lesions that, despite the relatively small numbers of animals, suggested something more than chance variation that might occur within a group. Six LAF_1's had severe cerebellar damage extending into lateral regions; the seventh was only somewhat less severe. Only two in each DBA group showed substantial cerebellar damage, and it was minimal in the others. Five LAF_1's showed considerable destruction of neurons and oligodendroglia in the striatum, while only one other mouse, a DBA/1, showed similar changes. All the DBA/1 animals showed the lateral necrosis of the medulla, but no DBA/2's showed it, and it affected one LAF_1.

Severe cortical damage was not frequent. Laminar necrosis of the neocortex (parietofrontal, Lamina II and III principally) occurred in three LAF_1's and once each in the DBA/1 and DBA/2 groups. Destruction of scattered neurons in the fascia dentata and pyramidal layers of the hippocampus occurred in a few DBA/1 and LAF_1 animals. Necrosis of oligodendroglia was more noticeable in the anterior dorsal thalamic nuclei of the LAF_1's than in the others. One LAF_1 (No. 16) and one DBA/2 (No. 14) showed widespread relatively massive regions of brain damage, including the preoptic-inferior septal-hypothalamic zones, and in the DBA/2 this extended to the mammillary bodies. The amygdaloid regions and inferior lateral striatum were severely affected in both of these animals, and they had severe extensive neocortical and olfactory lobe damage. The DBA/2 had only slight injury to the cerebellum, while in the LAF_1 it was severe and widespread.

One characteristic of the number of the lesions stood out in these experiments which had not attracted our attention before, namely, the sharp definition of many of the large lesions. Bilateral necrosis of virtually the whole colliculus in the midbrain, large bilateral regions of necrosis in the hypothalamus, sharply defined regions of necrosis in the cerebellum, and in many instances the bilateral complete necrosis of the lateral medulla were reminiscent of infarction. Figure 1 was selected to illustrate this phenomenon of sharp demarcation. No thrombosis or structural vascular damage was present, but dilated vessels were associated with these lesions. However, dilated vessels were frequent throughout these brains, probably as often in functionally and anatomically intact zones as in damaged ones. Some further suggestions about these lesions are given later.

Physiologic and Behavioral Responses.— In physiological and behavioral responses the C57's showed some considerable differences from the other mice. Only 1 of the 24 C57's died within moments of their irradiation, in contrast to the substantial numbers of immediate deaths in the other groups, and as a group C57's lagged behind the other strains in developing neurologic signs. In past experiments when similar large doses of radiation were given to C_3H, BAF_1, ABC, albino, black and tan, and yellow mice, and some others, one learned to expect that most of the animals would develop wobbly locomotion then become increasingly incoordinated and often finally be reduced to a state of complete incoordination and shaking movements. Sometimes the onset of wobbliness was quickly followed by a running fit and a general convulsion. Minor and major general convulsive bursts or thrashing movements were the rule when such animals were disturbed after the condition had developed. Few were able to walk about at all well. This syndrome, somewhat variable in any group of animals, began to affect some animals within a few minutes and was often fairly marked by half an hour and fully developed in two hours.

In the present experiments among the DBA's and LAF_1's these signs began to appear immediately after irradiation in some individuals and were well developed in most by two hours. Most of the C57's were virtually normal looking for the first two hours, and a number of them stayed that way longer. However, 15 C57's did die in the first 24 hours, 3 of them within

4 hours of irradiation and 7 more in 8 hours. Thus, while C57's were resistant to immediate death and developed neurologic disturbances slowly, death caught up with them later.

At the end of 24 hours there were still some differences in behavior among the strains, perhaps of some significance. Six of the seven LAF_1's were completely incoordinated and showed intermittent thrashing and convulsive movements. All seven DBA/2's were very quiet, but when disturbed, five thrashed around and showed general convulsive movements and the others were somewhat incoordinated. Three DBA/1's were markedly incoordinated and their hindquarters, virtually paralyzed. One was well enough to be eating, and another ate but walked in circles. The other two were quiet but thrashed about when disturbed. Of the 10 C57's, 1 (No. 25) dragged its hindquarters when it tried to walk, but it was not convulsive. Its lesions at autopsy were severe cerebellar and lateral medulla damage. Another walked in circles, holding its head to one side, but had no other obvious defect (No. 26). Its lesions resembled those of No. 25. A third (No. 30) and a fourth showed some incoordination when urged to move and general apathy. All four had the lateral medulla pattern of necrosis, and the third animal was the one C57 (No. 30) mentioned earlier that showed occasional necrotic cells in the cerebral hemisphere and diencephalon. Six C57's of the ten were rather unremarkable in appearance at 24 hours, and three of them were eating. The other four were as noted.

All of the 31 animals that were finally autopsied responded to sound, a hand clap 6 ft. away, by an immediate startled movement of the body. (The ears have not been studied microscopically, but severe cochlear damage was fairly frequent in past similar experiments.)

Comment

Three aspects of the results may be discussed. First, are there any real differences between the strains in responses to this massive dose of high-intensity radiation? Second, what is the nature of the well-demarcated lesions which remind one of infarction but which are not associated with vascular occlusion and do not clearly coincide with the distribution of arterial branches? Third, can the convulsions, susceptibility to immediate death, or other responses to irradiation be correlated with differences in brain metabolism known to exist between C57's and DBA's and alluded to in the introduction?

Differences Among Strains.—Three differences between the C57's and the other strains seemed to be clear. One was the resistance of C57's to immediate death from the high dose of radiation. A second was the lag of these animals behind the other strains in the development of the lethally convulsive state which was responsible for the death of animals during the 24 hours after exposure. The third was the conspicuous resistance to histologic damage of the cerebral hemispheres (except the olfactory lobes) in the C57 survivors as compared with the other strains. Whether these three are related to each other in any way will be commented on later.

Some other "differences" in patterns of lesions in the four strains appeared, and three may be recalled, for they suggest trends despite the small numbers. The DBA strains might have been expected to resemble each other, but all seven DBA/1's showed lateral medulla necrosis, while the seven DBA/2's did not. LAF_1's and C57's showed nearly consistent severe cerebellar damage, but the DBA strains usually escaped with very little. Of the four strains, the LAF_1's seemed to be most susceptible to several of the effects of radiation.

Demarcated Lesions.—The appearance of large, usually bilaterally symmetrical, lesions often as severe as an infarct and rather sharply defined suggested that local vascular factors might be responsible, but there was no mechanical occlusion, nor did the lesions seem to correspond obviously to arterial supply. Altered permeability of

regional vessels to substances usually withheld by blood-brain barriers was another possibility. Interference with venous drainage could be considered, but, as noted, dilated veins were not always related to the damaged zones. While neither vasospasm or vasoparalysis are excluded as factors, they would have had to act rapidly, for it is well established that the radiation necrosis is well developed as early as four hours (for example the DBA No. 15, killed four hours after exposure already had cerebellar and lateral medulla lesions, rare necrotic neurons, and oligodendroglia in the neocortex and occasional necrotic neurons in the inferior olive).

The often-observed fact that many of the damaging effects of radiation in given circumstances may be made worse by an increase in oxygen tension suggests the hypothesis that the zones of extensive necrosis may represent regions with relatively greater oxygen tension than their neighbors because of a relatively richer blood supply.[8] Some of the affected zones were ones with anatomically very rich capillary beds and overlapping tributary supplies (hypothalamic, cerebellar, collicular, neocortical Lamina II and III), while others were not (hippocampus). Regions with high oxygen tension could constitute loci minorae resistantiae with respect to radiation. A "rich blood supply" and correspondingly a "rich oxygen supply," however, is compounded of a number of factors, including rate and volume of blood flow and density and kind of capillary bed. Whether a high oxygen tension extracellularly and intracellularly generally would result would depend on how efficiently the oxygen supply and the oxygen demand of the local cells were balanced against each other in a given region. More data on these factors in various parts of the brain will be needed before this concept can be advanced further.

Genetic Patterns of Brain Metabolism and Radiation Damage.—One reason for carrying out these experiments was to see whether DBA mice with their deficient oxidative phosphorylating enzymes[3] and susceptibility to certain seizure-inducing factors were more prone to certain radiation injuries than C57's. Although the differences noted before were found, no simple formula emerges to explain them and there are no clear relations between the facts about the brain metabolism of the animals and their susceptibility to radiation effects. Nevertheless it is worth discussion on why some of the correlations failed to be realized and speculation on some others that may exist.

The immediate deaths may be reasonably attributed to radiation effects on the lower medulla, as demonstrated in previous experiments. Why the DBA's and LAF_1's were most vulnerable is unknown. Whether the convulsive deaths that developed in the next 24 hours represented a more slowly developing condition in the lower brain stem, with cardiorespiratory failure, convulsions, and death, or some other mechanism is also unknown. It is quite possible that these deaths were due to seizures resulting from forebrain damage and had nothing to do with the process of immediate medullary death. Cerebral edema and shock may occur after heavy irradiation and may have been contributory. It is not possible, either, to correlate the immediate deaths in the DBA's with the findings of Abood and Gerard,[3] who studied brain metabolism in DBA's. They found that the forebrain anterior to the level of the midbrain was the site of deficient oxidative metabolism when contrasted with that of C57's, while the brain stem was normal. If anything, C57's might have been expected to be vulnerable to death from radiation-induced fits because in previous experiments[2] they were more vulnerable to the lethal effects of methionine sulfoximine convulsions than the DBA's. The reason for thinking this was that general convulsions are known to exhaust brain energy stores very quickly, and C57's often died of cardiorespiratory failure during the first methionine sulfoximine-induced seizure. Paradoxically, DBA/2's withstood seizure after seizure

but developed forebrain lesions after the drug.

The patterns of destructive lesions were of little help in explaining the immediate death from irradiation. Indeed, many of the lesions were independent of the immediate deaths and convulsive episodes, and unless they affected a structure, alteration of whose function was rather obvious, they went unnoticed until autopsy. Nor was it possible to relate the metabolic deficiencies of DBA animals with the forebrain damage except to note their coincidence. However, if the speculation advanced earlier that regional hyperoxia may make a region relatively more vulnerable to the destructive effects of radiation on cells, then DBA animals might have such a condition in their forebrains, for it is conceivable that they have a regional disparity between the capacity of the blood supply to deliver oxygen and the tissue to use it.

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Delayed Effects of Ionizing Radiations in Man

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The discovery of x-rays and radium provided man with an important agent for use in investigative work and for use in medical diagnosis and therapy. However, soon after the discovery it became apparent that injudicious exposure to ionizing radiations could produce skin lesions of considerable severity. Later it was realized that repeated small doses of ionizing radiations or even a single large dose which may not in itself have caused signs or symptoms could after many years bring about serious injury in the area irradiated. It is about this second group of injuries, the long-term delayed effects, that this paper deals. The discussion is arbitrarily limited to those effects observed as the result of medical and occupational uses of ionizing radiations and does not include detailed consideration of the effects resulting from exposure to either the peaceful or military uses of nuclear energy. In the 63 years since the discovery of x-rays, the medical literature has become enriched with many case reports and comprehensive reviews of man's experiences with the adverse effects of exposure to ionizing radiations.

One very interesting fact about radiation-induced lesions is that the interval between actual irradiation and the development of disease manifestation in the area may be quite variable. On the cellular level, abnormalities in the mitotic cycle become apparent within minutes after exposure, but on the other hand, some of the tissue and organ manifestations may not become apparent clinically until an interval of many years has passed from the time of exposure to the time of manifestation of disease. This interval is popularly known as the latent period. It is a function of a number of radiation factors, the radiosensitivity of the tissue irradiated, and the age and the general state of health of the person irradiated. A good example of length to which the latent period may extend is found in a recent case report of a woman who at the age of 21 was subjected to a diagnostic roentgen examination for kidney stones lasting for about one hour. It was estimated that the skin dose was about 2000 r of 100 kv. energy. An acute dermatitis resulted after two weeks, but this healed. Later the skin became atrophic, but it was not until 49 years after the original examination that ulceration in the irradiated area occurred which on biopsy was found to be squamous-cell carcinoma.[82]

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Skin

The skin is a good tissue to consider in beginning a review of the delayed effects of exposure to radiation because it was reaction in the skin which made the early radiologists first realize that their new agent was not entirely innocuous. It was soon noted that even though the acute reaction in heavily irradiated skin tends to subside, the injured area shows gradually increasing evidence of atrophy of epidermis and fibrosis of dermis and subcutaneous tissues. Because the blood vessel walls become fibrotic with narrowing of the lumen, the blood supply to the injured area becomes so compromised that surface ulceration and deep-seated necrosis are apt to occur. The healing of such areas is prolonged, and infections, which are easily established, are difficult to cure. It has been the experience

of radiotherapists in recent years that doses of 1500 r are likely to cause permanent skin damage. Hair loss may be permanent with doses of 700 r or more. Sweat and sebaceous glands are destroyed with doses of about 1500 r. As the dose is increased above 1500 r the soft-tissue changes become more marked, until at the level of about 4000 r severe chronic radiation dermatitis is inevitable.[80] In skin with marked radiation changes, malignant lesions are likely to occur. The first radiation-induced skin cancer was reported by Frieben, in 1902 (quoted by Furth [38]). In 1911, 54 cases were reported in a monograph by Hesse (quoted by Furth [38]). The literature contains numerous reports of squamous-cell and basal-cell carcinomas developing in the skin of previously irradiated patients.[2,12,16,59,89,92,98,113,114] Acne, eczema, dermatitis, psoriasis, nevi, plantar warts, and a host of other benign skin diseases have been given radiotherapy, sometimes benefiting the original disease, sometimes not, sometimes with the development of chronic radiation dermatitis and subsequent skin cancer. Usually the cancer develops in an area of preexisting chronic radiation dermatitis, but not always.[114] Cancers develop in 20% to 30% of cases with chronic radiation dermatitis.[113,114] The latent periods have usually been long, on the average from 25 to 30 years, but may vary between extremes of 6 to 56 years.[14] An exception to the general rule was noted in a child who had received four x-ray treatments for an enlarged thymus gland shortly after birth. He presented at the age of 5 years with "several hundred" basal-cell carcinomas in the skin of the chest. In addition to the tumors, there was marked radiation reaction in the skin.[99]

Two recent reports have maintained that the use of low-voltage therapy in doses up to 1000 r is rarely if ever followed by significant adverse skin effects.[24,112] The follow-up periods in most instances in the two series of cases reported are too short to permit such conclusions to be drawn in spite of the fact that the classic stigmata of chronic radiation dermatitis have not been observed. There has been a tendency to underestimate the importance of radiation changes in the skin since the early days, and regardless of repeated warning signs to the contrary radiotherapy continues to be employed in the treatment of benign lesions often where some other therapeutic approach would be as satisfactory or even more effective.

Leukemia

It is generally accepted that leukemia can be induced in experimental animals by either a single large or multiple small exposures to ionizing radiations.[38,55] In addition to a great number of individual case reports, there are several large group or population studies which establish the fact that ionizing radiations may be leukemogenic for man also. The first of these is outside the scope of this paper but is noted for the sake of completeness. It concerns the follow-up studies on the survivors of the Hiroshima and Nagasaki atomic bombings in 1945 in which the incidence of leukemia has been shown to be greatly increased over that in the nonirradiated population, leukemia developing in 1 in 80 of those exposed within 1000 meters of the hypocenter.[83]

In addition to the single acute exposure which preceded by a period of one to six years the development of leukemia in the irradiated bomb survivors, chronic or multiple small dose, partial body exposure is also considered to be leukemogenic. The occupational exposure of radiologists, particularly in former days when the dangers were not as fully appreciated as they are now, is a case in point. It has been estimated by Warren that as short a time ago as the 1930's some physicians working with x-rays and radium may have received a total dose to large areas of the body of 1000 r or more.[120] Braestrup has calculated that the radiologists studied by Warren probably received an average lifetime dose of 2000 r in 40 years, while the earlier radiologists using nonprotective equipment probably received an average accumulated

exposure dose of 100 r per year.[11] An encouraging observation by Braestrup is that modern radiologists receive an accumulated dose of only about 1 r per year.

Radiologists have been found by many students of the problem to have a higher incidence of leukemia that their nonradiologist colleagues.[4,30,31,46,60,73,74,91,116] It was suspected even as early as 1912, when the first case of leukemia in a radiologist was published, that there might be a causal relation between exposure to radiation and the disease.[20] The total number of reported cases of leukemia in radiologists has been small; only 37 were found by March up to 1948,[74] and Lewis, collecting data from several sources, found that in the period from 1938 to 1952 there were 17 recorded deaths from leukemia among radiologists.[60] In spite of the limited numbers of cases, March was able to show that radiologists had about 10 times more deaths from leukemia than other physicians.[73,74] Warren made a comprehensive analysis of the causes of death of physicians as published in *The Journal of the American Medical Association* during the period from January, 1930, through December, 1954.[120] He was able to show that leukemia accounted for 3.65% of the deaths among radiologists but only 0.63% of the deaths among physicians who would not in the usual practice of medicine have been exposed to radiation. Lewis calculated the age specific death rates for leukemia for the white male population for 1938 to 1952 and compared these with the death rates from leukemia in radiologists. The increased death rates among the radiologists were considered to be statistically highly significant.[60]

As a full-scale expansion of a preliminary investigation,[22] a group study was undertaken by the Medical Research Council of Great Britain on 13,352 patients (11,287 men and 2064 women) who had been treated at 81 radiotherapeutic centers between 1935 to 1954 for ankylosing spondylitis.[23] Wide-field therapy was administered to the full spine and sometimes the pelvis in doses of 500 r to over 3000 r in one to eight courses. Forty-nine of the patients studied were found to have developed leukemia, aplastic anemia, or myelofibrosis. The 28 deaths from leukemia in this group were about 10 times the expected incidence in a healthy control population of the same age and sex distribution. Since the treatment is so widely used in Great Britain, only 400 untreated cases of ankylosing spondylitis could be found. These are too few controls to be statistically acceptable for a survey of this magnitude, but to date no evidence has arisen which suggests that ankylosing spondylitis is in itself a disease which is associated with a high incidence of leukemia.

When the incidence of leukemia in the male patients is plotted against the dose of x-rays calculated as integral dose (megagram-roentgens) and as mean dose to the spinal marrow, a relationship is found to exist which is curvilinear in the first instance, while in the latter it is a simple proportional one with lower doses but shows a disproportionately higher incidence with greater doses. Thus, the incidence increases from 0.5 per 10,000 men per year in the absence of treatment to between 16 and 17 cases per 10,000 men per year following a mean dose in excess of 1750 r to the spinal marrow. The incidence further increases to 72 cases per 10,000 men per year following a mean dose to the spinal marrow in excess of 2250 r. Two cases developed following doses of less than 500 r to the spinal marrow.

From Holland there is a preliminary report of seven cases of blood dyscrasias arising in patients with ankylosing spondylitis who had been treated two and one-half months to six years previously with x-radiation. Five of the cases are myeloid leukemias, two are aplastic anemias.[118]

Court Brown and Doll concluded that there is no evidence of the existence of a threshold dose of irradiation below which no increase in incidence in leukemia can be expected.[23] They further concluded that radiation in the amount of 30 r to 50 r

delivered to the whole red marrow of the body (93 r to the spinal marrow alone) was sufficient to double the spontaneous rate of leukemia. The question of the existence of a threshold dose is a controversial one of considerable importance, and opinions about it vary.[13,23,37,39,60,121] It has received much attention recently because of the slight addition to background radiation which has been contributed by world-wide fall-out of fission products. Also, its importance becomes apparent in cases in which treatment with relatively small doses of radioactive isotopes has been associated with the development of malignant disease. Three cases have been reported of acute leukemia arising in patients about 18 months after the treatment of toxic hyperthyroidism with 17 mc., 7.1 mc., and 2.1 mc. of I^{131}.[1,94,124] The total-body radiation delivered by the isotope amounted to only about 16 r, 7 r, and 1 to 3 r in these cases. It was concluded that the development of leukemia in these patients was entirely fortuitous. This seems justified, since it is not clear how doses of this low magnitude could induce leukemia, particularly after so short a latent period. There have been other cases, however, in which I^{131} has been used in larger doses in the treatment of thyroid carcinoma with development of leukemia four and five years after treatment was begun. The cause-and-effect relationship appears to be on a more sound basis in these cases, since the total-body radiation dose was estimated to be 600 r or more.[9,27,103]

It should be noted that other radioisotopes used therapeutically have been incriminated as agents capable of producing leukemia and other diseases, but there is no definite evidence in proof of the assumptions.[7,53,110] The 3% incidence of leukemia among 148 cases of polycythemia vera treated with P^{32} may well be the "natural" sequential development of the disease.[110]

A fourth study concerns the cause-and-effect relationship between thymic irradiation and the development of leukemia and thyroid cancer in children. Simpson et al. conducted a follow-up study on 1400 of 1722 children who had been treated in infancy with x-rays for thymic enlargement.[106,107] While in a comparable control group only 0.6 cases were expected (actually none were observed), 7 (or possibly eight) cases of leukemia were found among the treated children. Two of the treated children with leukemia had only 200 r or less exposure to the thorax, while the other five received 200 r to 1500 r. Although the number of cases is small, the fact that the incidence of leukemia is increased to more than 10 times the expected number is impressive. A survey now in progress at the Children's Medical Center in Boston approaches the problem from the opposite direction. It has been found that among 700 cases of leukemia 14 patients have a history of having received radiation therapy to the thymus gland.[34] This would suggest that perhaps 2% of childhood leukemias are caused by or are related to previous thoracic irradiation.

Thyroid Cancer

Duffy and Fitzgerald suggested that x-radiation might be an etiologic factor in the development of thyroid cancer in children.[32] Ten of their twenty-eight cases of thyroid cancer in children under 18 years of age were in patients who had received short courses of low-voltage x-radiation between the 4th and 16th months of life to relieve symptoms believed to be caused by enlargement of the thymus gland. Clark reported 15 cases of postirradiation thyroid cancer in children under 15 years of age.[18] Eleven of the cases who had previously been treated for thymic enlargement were known to have received air doses of from 200 r to 725 r in one to six treatments through ports measuring 3×3 cm. to 10×15 cm. The average latent interval was 6.9 years, with a 3- to 10-year spread. In the study of 1722 children treated with x-rays for thymic enlargement, it was clearly established that children so treated had an increased incidence of cancer of all types, 17 (or probably 19) being found, while the

expected incidence for a control group was 2.6.[106,107] The difference was particularly marked in respect to thyroid cancers, where the expected incidence was only 0.08, but 6 thyroid cancer cases were actually observed. The latent periods between time of irradiation and time of diagnosis of tumor ranged from 6 to 17 years. Majarakis and associates added 15 cases of cancer of the thyroid arising in patients between 5 and 20 years of age. Ten of these had received irradiation to the head and neck in doses of 200 r to 625 r in infancy or childhood.[72]

Cancer of the thyroid is an infrequent disease in children,[49,122,126,127] but the implication of irradiation as an etiologic factor is strongly supported in many papers.[18,28,32,35,72,126,127] It is interesting to note that in his definitive review of cancer of the thyroid in children for the period from 1900 through 1950, Winship was able to establish the time of onset of the disease in 169 of the total of 191 cases reported. By decades, the number of cases increased from 1 in the first (1900-1910) to 4 in the second, 13 in the third, 30 in the fourth, and 121 in the fifth. Ninety of the one hundred twenty-one cases in the last decade appeared in the period from 1946 to 1950.[126] There is a history of thymic irradiation in approximately 20% of the collected cases of thyroid cancer in children in the United States.[127] Irradiation of the thymus gland in infants under one year of age was a commonplace procedure in this country from 1930 to 1945 and is still practiced in a few communities. Since the latent period for development of thyroid cancer ranges from 3 to 17 years, it may be difficult to detect a change in trend of the numbers of thyroid cancer cases in children until after 1960, but after that date if thymic irradiation in infancy is an etiologic factor of importance, the number of new cases, corrected for population growth, should decline.

Congenital and Developmental Hazards

It is encouraging to note that the hazards of radiation in children are widely publicized. The specific tissue effects are much as those in adults, but in addition special attention must be paid to the avoidance of growth disturbances, both acute and delayed,[26,57] and to the avoidance of genetic damage.[95] The importance of limiting radiological procedures to those that are clearly indicated and then only where adequate gonadal protection is used cannot be overemphasized. The genetic hazard is one not only to the individual person but to the population as a whole. Understanding of this hazard and the exercise of rigorous discipline on the part of pediatricians in avoiding gonadal radiation of their patients is, indeed, vital to the future of the human race.

That congenital malformations can be induced by pelvic radiotherapy in a female carrying a developing embryo in utero is well known.[42,70,84] Recently the possibility has been suggested that diagnostic radiation procedures carried out on the pregnant female may cause the development of malignant disease in the child who had previously been irradiated in utero.[109] The histories of 547 children who died before the age 10 with leukemia and other malignant diseases were reviewed. Of the 269 cases of leukemia, 42 cases were in children born to mothers who had received diagnostic x-ray examination of the abdomen in the prenatal period. Among 269 healthy controls, 24 children had been subjected to similar irradiation but did not develop leukemia. Of 278 cases who developed other malignant diseases and 278 healthy controls, the history of prenatal exposure to abdominal x-ray examination was present in 43 of the cancer cases but in only 21 of the healthy controls. It is estimated that the fetus receives 8 r to 12 r in the course of the usual abdominal x-ray examination of the mother. This is a small amount of radiation, and these data are far from conclusive, but they are suggestive enough to warrant continued

and more comprehensive investigation. The Armed Forces Institute of Pathology is now in the process of requesting the hospitals and physicians of this country to supply pertinent data for analysis and comparison with the British experience.

Pelvic irradiation before conception has been an accepted treatment for relief of infertility and menstrual dysfunction.[56,96] While follow-up studies on the children and grandchildren of some of the women who had this treatment fail to show clinical evidence of gene mutations, it should be realized that at best only a small fraction of the total genetic damage could be detected in the short time elapsed and the small population available for study.

Respiratory System

Both therapeutic and occupational exposures to ionizing radiations have sometimes resulted in undesirable delayed effects involving the respiratory system. These range from pulmonary fibrosis with reduction of respiratory reserve and increased susceptibility to infection, which may follow exposures of the order of 2000 r,[86] all the way to bronchogenic carcinoma, which has been a common disease among certain miners for many years.[62,90,93,105] The mining and milling of uranium ores in the United States is a recent industrial venture, but a public health study has indicated that in the unventilated mines of the Colorado plateau the concentration of radon is higher than the median concentration reported in the mines of Bohemia and Saxony.[29] To date, lung cancers in American uranium miners have not been reported, but it is known that they excrete polonium in their urine.[111] In the pitchblende mines of Bohemia and Saxony, the miners, who often begin their labors as boys, frequently develop "miner's disease," characterized by cough, hemoptysis, and shortness of breath. In the past, many miners died in early middle age, 15 to 20 years after starting to work in the mines. Subsequent investigations, though incomplete, suggest that about 50% of the deaths were due to lung cancer.[90,93,105] Radon as such and the particulate radioactive daughter products of radon which become adherent to dust are inhaled. The radioactive dust, because of difference in sizes of particles, lodges in different parts of the bronchial tree, where some is removed by physiologic mechanisms but some becomes "fixed." That which is retained subjects the adjacent tissue to intense α-radiation over long periods of time. Evans calculated that the radon concentration in the mine air of 3×10^{-9} curies per liter would, over the average tumor induction time of 17 years, deliver about 3000 r to the site of the tumor.[33]

Lesions in Dial Painters and in Patients Treated with Radium Salts

During the period from 1914 to 1925 it was customary for luminous-watch-dial painters to achieve a fine point on their brushes by compressing them between tongue and lips. Varying mixtures of radium, mesothorium, and radiothorium were used in the luminous paint and were absorbed by the painters in the process of "tipping" their brushes. By 1925 it became evident that this habit was responsible for much of the illness and death in these workers.[17,75] A small portion of radium and thorium compounds found their way to the bones, where they tended to remain for many years, meanwhile subjecting the surrounding tissue to intense α-radiation.[66]

During this same era, the administration of radium salts by mouth and parenterally was a popular form of therapy for a variety of diseases, ranging from dementia praecox[88,100] to tuberculous arthritis.[5,6,45,87] Other diseases which had the benefit of this therapy include hypertension, cardiovascular disease, duodenal ulcers, gout, neuritis, diabetes, Buerger's disease, syphilis, infected wounds, skin diseases, anemia, leukemia, and nephritis.[64]

About 5 to 10 years after the first medical and industrial exposures to radium, some of the radium-containing patients began to die

TABLE 1.—*Tumors in Man from Ra Poisoning* *

Type of Tumor	Ra, μg.	Exposure Type	Death, Yr.†
Osteosarcoma, humerus	8-9	Therapeutic injection	23
Osteosarcoma, tibia	6.5	Dial painting	27
Osteosarcoma, finger	0.8	Therapeutic injection	28
Gaint-cell sarcoma, jaw	1.5	Dial painting	15
Fibrosarcoma, knee joint	5-6	Therapeutic, oral	17
Squamous carcinoma, sinus	10	Dial painting	19
Squamous carcinoma, sinus	2-9	Dial painting	34
Squamous carcinoma, sinus	1.0	Dial painting	34
Osteosarcoma, femur	3.8	Dial painting	32
Osteosarcoma, elbow	0.5	Dial painting	Living
Osteosarcoma, ischium	1.3	Dial painting	30
Fibrosarcoma, foot	1.3	Therapeutic injection	22
Osteosarcoma, tibia	0.8	Therepeutic injection	24?
Osteosarcoma, vertebrae	6.8	Therapeutic injection	?
Osteosarcoma, tibia	3.6	Therapeutic injection	24

* According to Looney, cited in reference 41.
† After exposure.

with severe anemia, aseptic necrosis of bone, and bone tumors. The publications of Martland [75-79] and others [10,10a,17,36,48a,101,102] alerted the world to the dangers inherent to the use of and exposure to radium. Investigations were carried out in 1952 [3] and later [64-66,69] on a total of 50 patients who had received radium salts therapeutically and 28 who had been employed in the luminous-dial industry. The average time of retention of the radioactive material in the 78 patients was 25 years, with limits of 10 to 35 years. Although Aub et al. cited cases of three patients with a 20- to 25-year body burden of radium of 10μg. to 23μg. who had no serious interference with good health, one case with as little as 0.5μg. had evidence of radiation-induced disease. (Some mesothorium and radiothorium contamination in this case was not included in the determination of body radioactivity. Had these amounts been added, the total figure would be something more than 0.5μg.) Eleven patients with asceptic necrosis of the femoral head had 0.7μg. or more for an average retention time of 15 years. Fifteen malignant tumors developed in the 78 patients, a 14% incidence. Eleven of the tumors were sarcomas of bone; one, a fibrosarcoma of the knee joint, and three, squamous-cell carcinomas of the nasal sinuses (Table 1). The patients carried between 0.5μg. and 10μg. of radium for an average retention time of 25 years. From these data Looney calculated the accumulated radiation dose (rad) from radium required to produce major skeletal damage and bone tumor formation to be in the order of 1000 to 2000 rad during the 25 years.[66]

The studies of radium deposition assume great significance if it is recalled that 0.1μg. is the maximum permissible concentration (MPC) of radium.[85] This amount of radium maintained over a lifetime from 15 to 70 years of age will provide an estimated accumulated skeletal dose of 200 r. It follows that if the MPC is exceeded by 5 to 10 times the accumulative dose capable of carcinogenesis is achieved. This is a rather narrow margin of safety, but it is considered to be adequate at present.

Lesions in Patients with Retained Colloidal Thorium Dioxide (Thorotrast)

Interest has recently been revived in the cancerogenic properties of retained Thorotrast, a 20% solution of thorium dioxide.[44,54,67,68] Thorotrast is radiopaque but is also an α-emitter with a long half-life. It is picked up by the cells of the reticuloendothelial system and is stored and concentrated in them either locally or, when administered intravenously, chiefly in the liver and spleen. It was introduced in 1928 and became widely used in succeeding years, the intravenous dose varying from 3 to 15

TABLE 2.—*Leukemias and Malignant Tumors After Injections of Thorotrast* *

Disease	Organ	Latent Period, Yr.
Myeloid leukemia	Marrow	6
Myeloid leukemia	Marrow	10
Lymphatic leukemia	Marrow	10
Stem-cell leukemia	Marrow	14
Stem-cell leukemia	Marrow	7
Sarcoma	Kidney	16
Carcinoma	Eyelid	?
Carcinoma	Eyelid	?
Carcinoma	Max. sinus	10
Carcinoma	Breast	10
Endothelial sarcoma	Liver	3
Endothelial sarcoma	Liver	12
Endothelial sarcoma	Liver	23
Hemangioendothelioma	Liver	14
Hemangioendothelioma	Liver	14
Hemangioendothelioma	Liver	19
Hemangioendothelioma	Liver	23
Carcinoma	Liver	?
Carcinoma	Liver	19
Cholangioma	Liver	10

* According to Looney et al.[68]

gm. The first well-documented case of a neoplasm following the intravenous use of colloidal thorium dioxide was published in 1947.[71] The patient developed an endothelial sarcoma in the liver near colloidal thorium dioxide deposits 12 years after receiving the contrast medium intravenously in the course of a diagnostic procedure. A variety of soft-tissue tumors both carcinomas and sarcomas have since been reported and related to previous colloidal thorium dioxide injections (Table 2). Evans has calculated that tissue containing 1% colloidal thorium dioxide would receive approximately 3000 rad in 10 years.[33]

Bone

Benign and malignant lesions of bone may be produced by externally applied irradiation as well as by irradiation from internally deposited radioactive materials. In 1948, Cahan and associates reviewed the literature on the subject and added 11 cases of their own.[15] They were able to find 14 cases from the European countries and a few isolated case reports from this country. All of the European cases developed in bones being treated for suspected or proved tuberculous arthritis. One of the patients had received intra-articular radium chloride injections, but the others had received external irradiation. The latent period varied from 3 to 12 years, with an average of 6.2 years.

In the cases of Cahan and associates filtered x-rays generated at 130 kv. to 200 kv. were used to deliver total doses accumulated over weeks or months, ranging from 1550 r to 25,000 r. Benign lesions were treated, but in both normal and diseased bone sarcomas developed in from 6 to 22 years. Sabanas and associates added 17 cases who after x-ray alone (13 cases), radium alone (1 case), or a combination of the two (3 cases) developed sarcomas of bone.[97] The tumor doses ranged from 1400 r to 10,000 r given in single or repeated courses spread over periods of up to 39 months. The latent periods extended from 32 months to 30 years, with an average of over 10 years. According to Vaughn, who reviewed the literature in 1956, thirty-nine cases of bone sarcoma following radiation had been reported.[119] Cruz and associates, in 1957, added 11 new cases in persons who had received 100 kv., 250 kv., and 1 mev radiation in amounts of 1000 r to 5289 r given over a period of one month to nine years.[25] Osteogenic sarcomas developed in previously normal and previously diseased bone in 4 to 24 years after exposure.

Nasopharynx

Radiation of the cervical lymph nodes in the treatment of tuberculous adenitis and of the thyroid gland in the treatment of thyrotoxicosis has been associated with the subsequent development of several squamous-cell carcinomas of the larynx and nasopharynx and of two carcinomas of the thyroid.[14,43] The latent period is usually long, 10 to 30 years, and the number of cases reported so far is few. The practice of shrinking lymphoid tissue about the Eustachian tube ostia by radon applications in order to allow submariners to equalize middle ear pressure rapidly was routine where "indicated" as recently as 1951.[125]

This treatment was also given to pilots in the United States Air Force.[50] Although doses were usually small, in some instances the mucosa adjacent to the ostia has become scarred and shows evidence of chronic radiation injury.[55] Service men who had the treatment should as a safety measure have periodic examinations of the nasopharynx for at least a 30-year follow-up period.

A consultant in *The Journal of the American Medical Association* for Oct. 13, 1951, recommended 300 r to each of two parts, given in doses of 100 r weekly, for the treatment of enlarged adenoids. The treatment was suggested as being particularly useful in children when surgical removal of the adenoids was not feasible.[128] Undoubtedly a number of patients received such treatment, and, although they may have experienced less localized tissue damage than those patients who were treated with radon, they too should be carefully followed for possible delayed effects.

Female Pelvic Organs

Ionizing radiations have been used for functional uterine bleeding, infertility, castration, and other benign diseases of the pelvic organs. In a group 958 patients given radiotherapeutic menopause, mostly by intrauterine radium insertion, and followed for an average of 6.7 years, it was concluded that the nine cancers of the uterine corpus in the group (with only six in the cervix) developed coincidentally and not as the result of radiation.[21] Speert, in reviewing the same group of 958 patients, contended that coincidence was difficult to accept as the complete explanation for the development of the cancers of the uterine corpus.[108] However, he was able to conclude unequivocally that ionizing radiations as used for benign gynecological conditions were not tumorigenic for the human ovary.[108]

In another study of 500 cases of endometrial carcinoma, 32 patients had endometrial biopsies 1 to 23 years prior to the diagnosis of carcinoma. Twenty of the thirty-two cases received radiation therapy for benign conditions of the uterus seven to eight years before the diagnosis of cancer was made. It was concluded that the incidence of cancer was greater than could be expected in an untreated population.[47] Of particular interest was the development of 3 carcinosarcomas among the 20 irradiated cases. This is an unusually high incidence of this relatively rare tumor type, and investigators are in general agreed that a cause-and-effect relationship with prior irradiation probably exists with respect to this cancer,[47,48,108] although the relationship is equivocal with respect to development of adenocarcinoma of the uterus.[123]

Late Hematologic Effects

Except for the leukemias and the occasional aplastic anemia, which were discussed elsewhere in this report, the late hematologic effects of exposure to ionizing radiations are difficult to quantitate. There is general agreement that the hematopoietic cells are radiosensitive and that both large doses and repeated small doses of radiation will in time cause a depression of the bone marrow which may be manifest as a decreased cellularity [51] or as a hyperplasia of reticuloendothelial cells, with failure of maturation.[61] Lymphoid tissues undergo a similar depletion. In time the changes in the hematopoietic centers are reflected in the peripheral blood.

Ingram has recently summarized the literature in this field and was able to collect a surprisingly large number of well-documented cases in man where the degree of exposure was known with some accuracy.[52] It is interesting to note that chronic exposures with 200 kvp, 400 kvp and 1000 mev x-rays in the amount of 40 r delivered to the total body may cause a persistent lymphopenia (Low-Beer and Stone, cited by Ingram [52]). Similarly, the calculated 175 r total-body dose from radioactive fallout sustained by the Rongelap natives in 1954 resulted in decreased lymphocytes, monocytes,

eosinophils, and platelets in peripheral blood. The depression was still evident one year after the exposure (Cronkite et al., cited by Ingram [52]).

Although the degree of exposure of the persons is often not known and it is often difficult to be certain of a change in peripheral blood, particularly at low dose levels, it seems fair to say that the constituents of peripheral blood are probably very sensitive indicators of radiation injury. The great body reserves and the defense mechanisms of the body make the task of demonstrating slight changes very difficult, especially since our diagnostic methods are crude and subject to error.

For a comprehensive bibliography on the effects of radiation on the blood and blood-forming organs see Reference 86.

Cataracts

Radiation is capable of interfering with the maturation of the lenticular cells of the eye and of destroying their anterior layers. Incipient cataracts develop about three to six months after exposure to sufficient amounts of radiation. Tuttle estimates that in the adult human lens x-ray exposures in excess of 2000 r are necessary to produce cataracts.[115] However, other investigators report cataract formation with x-ray doses ranging from 200 r to 1000 r.[19,81] Furthermore, cataracts have been said to occur in all patients who receive lens doses in excess of 1150 r, regardless of the duration of the treatment.[81] Neutrons are considered to be about 10 times more effective in producing cataracts than are x-rays. Among the 8000 exposed Japanese survivors, there have been only 10 cases of severe cataracts, 25 cases of impaired vision due to posterior polychromatic plaques, and about 200 cases with minimal lenticular changes.[115]

Shortening of Life Span and Aging

The effect on shortening of life span of single doses of radiation and multiple doses or chronic irradiation has been investigated experimentally.[8] Both methods of exposure are effective in shortening the life span of animals in amounts which vary with the size of the dose and with the dose rate. For reasons not understood, a single large dose is more effective than multiple small doses of the same aggregate size. Paradoxically, however, at levels of radiation of 0.1 r daily throughout their life, the life span of mice may be lengthened by as much as 10%.[63]

It would appear from the analysis made by Warren of 82,441 physicians, relating their age at death to their specialty, that radiation is also associated with shortening of life span in man.[120] He found that radiologists die younger than do nonradiologists from every major cause of death, with the exception of anemia. Radiologists as a group died 5.2 years earlier than nonradiologists. This amounts to 11% of the adult life span (after age 20).[86] On the basis of a 1000 r to 2000 r life-time exposure, each exposure unit may be said to be responsible for one to two days' loss of life span.

Experimental work with animals [8,117] supports Warren's contention, but there has been some objection to Warren's interpretation of the human data by statisticians who have analyzed the data on the basis of age specific death rates.[60,104] It is said that radiologists "would be expected to die at younger ages, because there are proportionately fewer elderly radiologists." [104] This does not prove that exposure to ionizing radiations does not have a life-shortening effect but does indicate that differences in age composition alone could account for the apparent decrease in life span noted in radiologists compared with nonradiologists.[104]

This process of aging is inevitably an inherent factor in limiting life span. It is possible that the shortening of life span which appears to be associated with exposure to radiation is the result of speeding up of the aging processes.[117] The radiation effect on blood vessels and connective tissues certainly suggests that this may be a factor, but as yet our ability to measure and interpret minimal evidence of deterioration

is not sufficiently developed to allow definite conclusions to be drawn.

Conclusions

Any given response to irradiation is the result of the interplay of many physical factors which acting together characterize the agent and many biological factors which acting together determine the host's response to the agent. A great deal is known about the physical factors, and the agent can be more or less tailored to desired specifications. The host's response and the finer details of biologic mechanisms are, on the other hand, shrouded in mystery and are not often in our present state of knowledge subject to effective alteration. Some of the delayed effects of exposure to ionizing radiations may be so slight that they are not recognized and their importance is not appreciated. This is particularly true at low dose levels, where the defense and repair capabilities of the body may be sufficient to mask or reverse smaller degrees of damage. The struggle may be prolonged, but in time if the damage has been severe enough and the host's response not effective enough, the injury becomes manifest. A long, usually symptom-free, latent period is characteristic of many radiation-induced lesions. The total number of known cases in most categories of delayed effects is small, and the amount of radiation required to produce the more serious effects is large. Radiation-induced malignant diseases number fewer than 100 cases in each of the several categories, and the induction doses are usually 1000 r at a minimum but on the average are nearer 3000 r.

The important thing to recognize is that serious delayed effects in man can be caused by exposure to ionizing radiations. In the past significant exposures have been sustained because of ignorance, carelessness, and accidents. There is still much to be learned about low and cumulative dose effects and about the existence of a threshold. Great strides have been made in the understanding of protective devices and in generating an awareness of the potential dangers in unwise use of ionizing radiations. As agents in diagnosis and therapy they have saved and prolonged life and immeasurably facilitated the practice of medicine. They have opened great new fields of medical, biological, and physical sciences research. As man enters the space age, he will come to rely more and more on nuclear energy to supply power and industrial needs. More persons employed in atomic power plants, in uranium mining and processing, in the production of isotopes, and in the scientific and industrial applications of ionizing radiations will obviously put more persons at risk. Accidents will occasionally occur, but the proper use of protective devices will minimize the hazards. Physicians must also take steps to minimize the hazards. The careful use of protective devices and the avoidance of unwarranted use of even the slightest amount of ionizing radiations are the hallmarks of enlightened modern medical practice. There is no cause for curtailment of any clearly indicated medical use of ionizing radiations, but, particularly in children, young adults, and pregnant women, and in benign diseases, the indications for their use must be carefully reviewed, appraised, and justified.

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A New Technique for Ultraviolet Microbeam Irradiation of Living Cells

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The concept of utilizing a microbeam of ultraviolet light for the production of ultraviolet irradiation damage in selected areas of living cells is attributed to Tschachotin, in 1912.[1,2] Tschachotin achieved an ultraviolet microbeam by means of bringing to microscopic focus an ultraviolet light source, using refracting lenses as the focusing elements. His technique suffered principally from the difficulties involved in the proper aiming of the spot and hence in achieving focal irradiation of selected small areas of cells. In 1954, Uretz, Bloom, and Zirkle reported a much improved method of obtaining an ultraviolet microbeam.[3] Their method had the advantage that the microbeam could be focused and aimed through the microscope. Thus one could visually select by means of focused cross hairs the precise spot of microbeam irradiation which the specimen in view was to receive. An ultraviolet microbeam of approximately 7μ in diameter may be achieved by this method. After the delivery of the focal irradiation, time-lapse phase-contrast motion pictures of the specimen or one or two ultraviolet-absorption images of the specimen may be obtained. Ultraviolet time-lapse motion pictures could not be obtained because of the prohibitively intense doses of ultraviolet necessary to obtain such motion pictures.

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The purpose of this communication will be to report a new method of achieving an ultraviolet microbeam for the purpose of producing highly selected areas of ultraviolet irradiation damage in living cells.

The basis for this technique is formed around the concept of the ultraviolet flying-spot television microscope. The general principles of this instrument have been previously reported by us.[4]

Briefly, the ultraviolet flying-spot television microscope functions as follows: A scanning spot of ultraviolet light traces out a rectangular pattern on the face of an ultraviolet-emitting cathode ray tube. This pattern is termed a raster and in this application represents the light source. This raster is derived from two saw-tooth voltages which determine the horizontal and vertical trace velocities of the scanning spot and thereby determine the number of the lines in the raster and its repetition rate. In practice this raster consists of a rectangle of lines approximately 1.25 in. in height and 1.75 in. in width. Within this area the spot traces out 250 individual lines. This number is arbitrary and may be increased or decreased to meet the resolution limitations of the spot size of any given cathode ray tube. The total raster build-up time may be adjusted from 1/20 of a second to 10 seconds. The repetition rate of the raster may be adjusted from 1/20 of a second to several hours. The over-all brightness of the raster may be varied by altering the grid-to-cathode potential of the cathode ray scanner tube. Accordingly, selected areas of the raster may be intensified or extinguished by applying a rectangular voltage pulse of suitable phase and amplitude between the grid and cathode of the scanner tube. This pulse is obtained from two pulse generators; one, arranged to generate a pulse of variable width when the horizontal saw-tooth has reached a predetermined voltage level, and the other, to perform a similar function when the vertical saw-tooth generator has reached a predetermined voltage level. The outputs of these pulse generators feed a co-incidence detector, and the intercept of the horizontal and vertical pulses is amplified and brightens the scanner tube raster. Adjustment of a vertical delay

control moves the brightened area up or down the scanner-tube raster. Adjustment of a horizontal delay control moves the spot from one horizontal position to another on the scanner-tube raster. Adjustment of either pulse width will change the dimensions of the brightened area. The brightened area may be from one to several hundred picture elements in size and may be variously adjusted in intensity according to the experimental need. The converse of this situation may also be obtained by extinguishing the spot rather than intensifying it. This will produce an area in which no ultraviolet irradiation is taking place rather than an area in which intensified irradiation is being given.

The image of the raster on the face of the scanner tube is demagnified by a quartz eyepiece and a reflecting objective focused on the specimen. A 45 degree first-surface minor mounted above the eye piece permits the microscope to be mounted conventionally. In this manner the living cell specimen is scanned by a microspot of radiant energy whose dimensions will be given by the initial spot size, the power of the optical components, and the optical throw distances. The spectral characteristics of the spot are determined by the emission spectrum of the scanner tube modified by suitable reflectance or interference filters interposed between the specimen and the scanner tube. The radiation transmitted by nonabsorbing or lightly absorbing areas of the specimen is collected by a quartz condenser and directed onto the photocathode of an ultraviolet sensitive photomultiplier tube. The output of this tube feeds a videoamplifier, whose band width is adjustable to cover the range of scanning speeds and for which γ-correction is provided. The output of the amplifier drives a monitor employing a P19 phosphor of the type commonly found in radar tubes. Since the scanner tube and the monitor tube are in synchrony, a magnified visible image of the ultraviolet absorption pattern of the specimen appears on the radar monitor. The modulation of the monitor tube thus represents the modulation impressed on the ultraviolet output of the scanner tube by the instantaneous absorption of the specimen at each image point. At the end of each complete raster build-up a pulse is generated; this pulse is differentiated and switched alternately to two preset counters. The counter outputs then control precisely the lengths of the photographic exposures and intervals. In this way time-lapse motion pictures of the ultraviolet absorption images of living cells may be obtained. Figure 1 is a general photographic view of the ultraviolet flying-spot television microscope, with the accompanying equipment for time-lapse motion picture photography. The pulse generators which produce and control the microbeam may be seen on either side of the viewing monitor in the upper center. The ultraviolet-emitting cathode ray scanner tube may be seen just between the two meters below the viewing monitor. The reflecting microscope is housed in the incubator just below and in front of the ultraviolet scanner tube. The synchronized time-lapse motion picture equipment may be seen on the right. Figure 2 is a block diagram of the equipment and is self-explanatory.

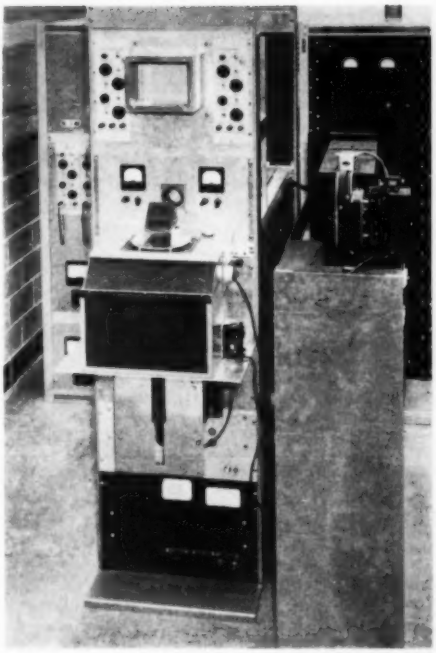

Fig. 1.—A general view of the ultraviolet flying-spot television microscope. The pulse generators for microbeam production and control may be seen on either side of the viewing monitor top center. The ultraviolet scanner tube may be seen between the two meters below the monitor tube. The microscope is housed within the incubator just below the scanner tube. The time-lapse motion picture equipment may be seen at the right.

By thus making use of the principle of scanning spot illumination and the employment of a photomultiplier tube for image conversion, ultraviolet absorption images of living cells may be obtained over long periods of time without evidence of cellular damage. As previously reported, marked cellular damage may be produced by brightening the entire scanner-tube raster.[5] With this technique irreversible damage may be produced in less than one hour of scanning.

With the recent provision for microspot intensification or extinction, a variety of

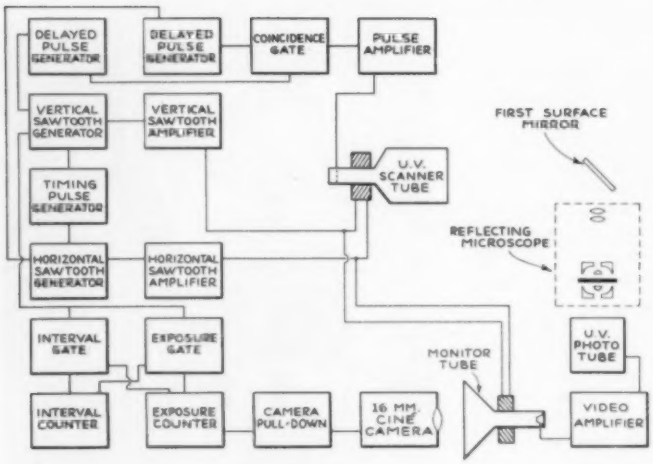

Fig. 2.—Block diagram of variable sweep-speed ultraviolet flying-spot television microscope with provision for microbeam irradiation.

hitherto impossible ultraviolet irradiation studies of living cells may now be achieved. For example, any area of the specimen down to 1μ in size may be intensely and virtually continuously irradiated while nondamaging levels of irradiation are applied to the remainder of the specimen for the purpose of simultaneously recording the ultraviolet absorption images of the specimen by time-lapse motion picture photography. Thus one could intensely irradiate an entire nucleus, or an entire nucleolus, or a portion of a nucleolus down to 1μ in size while recording the accompanying absorption changes in the damaged and undamaged portion of the specimen by time-lapse motion picture photography. Figure 3 is a photograph of the screen of the monitor tube, upon which is displayed the ultraviolet absorption image of a living HeLa cell. The brightened spot in the picture represents a focal area of intensified ultraviolet irradiation being delivered to the nucleolus of the cell. In this case the microspot is approximately 1μ in diameter. The converse of this irradiation technique may also be accomplished by intensely irradiating the cytoplasm while delivering a nondamaging

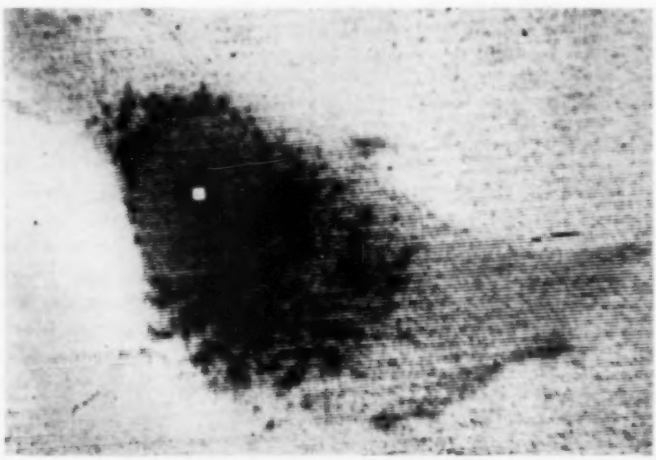

Fig. 3.—Ultraviolet absorption image of a living HeLa cell. The brightened spot centered in the nucleolus represents a 1μ square microbeam of ultraviolet irradiation.

level of irradiation to the nucleus, or, in terms of smaller areas, intensely irradiating the entire specimen with the exception of the nucleolus, or an area in the nucleolus down to 1μ in size. This technique therefore offers the possibility of studying the relationship between ultraviolet irradiation damage of the total specimen and ultraviolet irradiation damage of highly selected areas of the specimen and, in addition, of being able to simultaneously record by time-lapse motion picture photography the resulting absorption changes in both the damaged and the undamaged portions of the specimen. At the present time no other technique is available for such studies.

Summary

A new technique for microbeam ultraviolet irradiation studies of living cells is described.

This technique permits ultraviolet irradiation of selected areas of the living cell specimen down to 1μ in size.

The converse of this technique makes it possible for the entire specimen to be intensely irradiated while any area down to 1μ in size is excluded from intense irradiation.

In conjunction with the intense microbeam irradiation time-lapse motion picture studies of the resulting ultraviolet absorption changes in both the damaged and undamaged areas may be recorded.

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Pulmonary Hyaline Membrane

An Experimental Study

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Numerous recent papers have tried to explain the chemical nature and pathogenesis of hyaline membranes in the lungs of the newborn infant. Many different substances have been injected intratracheally in attempts to reproduce them. Generally amniotic fluid has been used; it has usually failed.[1] Farber and Wilson,[2,3] using India ink, horse serum, hydrochloric acid, and fibrinopurulent exudate, had some measure of inconstant success. Tran-Din-De and Anderson,[1] in a review of the subject, reported intratracheal injection of various substances, such as cadmiun chloride, vernix caseosa, meconium, Ringer's solution, and isotonic saline solution, with negative results. Oxygen poisoning, CO_2 poisoning, and irradiation have caused formation of hyaline membranes, but not consistently.

Laufe and Stevenson[4,5] were able to produce membranes by intratracheal injection of human plasma plus amniotic fluid in 6 out of 34 guinea pigs; with repeated injection of the same material they obtained a higher incidence of success—3 out of 12 guinea pigs.

Experiments undertaken to ascertain the composition of the hyaline deposit have led to a great variety of conclusions and frequent contradictions. More definitive results were reached by Gitlin and Craig,[6] who demonstrated an abundance of fibrin in the membrane by using a fluorescein-labeled fibrin antibody. Later, the same authors[7] showed the reason why histochemical reactions for fibrin fail at times, thus explaining contradictions found in the literature.

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Neustein and van Breemen[8] and van Breemen, Neustein, and Bruns,[9] on the basis of purely morphologic studies with the electron microscope, concluded that the hyaline membrane consists of a mass of fibrils, representing fibrin, cell debris, and plasma proteins.

The present investigation had for its purpose a search for a substance or substances capable of forming a hyaline layer in the pulmonary alveoli. The study is limited to substances which could appear in the lung or could be introduced into it under natural conditions and excluded extraneous material, such as cadmium chloride and mustard gas, which could reach the lung only by artificial means.

This paper does not try to elucidate whether the hyaline membrane is endogenous or exogenous in origin.

Materials and Methods

A total of 60 young male and female rabbits, weighing between 500 and 1500 gm. was used. They were given injections intratracheally of different substances in amounts varying from 5 to 15 ml., depending upon the weight of the animal, its individual susceptibility, and the type of substance. Eight animals died during the injection, one at the moment of puncture, the remainder after 10 ml. of fluid had been instilled. These animals are excluded from our series. All the animals were given penicillin intramuscularly for two consecutive days following the intratracheal instillation.

They were killed in groups of two every 24 hours. The respiratory tree was fixed in buffered formaldehyde solution. Five sections were taken at different levels from each lung of every animal; the sections were embedded in paraffin and stained with hematoxylin and eosin.

Results and Comment

The substances used and the corresponding results are listed in the Table.

Substances Used and Results

Group	Substance Injected	Animals Treated, No.	Cases with Hayaline Layer, No.	Extent of Lesion
I	Control; saline solution, 0.7%	5	0	
II	Human plasma, single injection	6	1	+
III	Human plasma & calcium chloride, equal parts	23	23	++++
IV	Human amniotic fluid from Cesarean section	12	3	+
V	Human amniotic fluid & calcium chloride	8	0	
VI	Same as Group V, plus oxygen chamber for 24-48 hr.	6	0	
VII	Human plasma from same person, 3 injections at 1, 30, & 45 days	7	3	++

These substances were selected for injection after taking the experience of other investigators into consideration. Isotonic saline solution was instilled intratracheally as a control in five animals (Group I); none of these developed a hyaline membrane.

Human amniotic fluid was used in Group IV, with practically negative results. Human amniotic fluid mixed with 0.22% calcium chloride was used in an attempt to produce intra-alveolar coagulation of the injected substance; the results were equally unsuccessful (Group V).

The animals in Group VI received the same mixture of amniotic fluid and calcium chloride but in addition were then placed in a closed chamber under a constant stream of oxygen from 24 to 48 hours. No hyaline membrane was formed; indeed, of all the animals, this group showed the least inflammatory reaction.

Through a mistake that occurred at the beginning of these experiments, one rabbit received a second injection of human plasma 10 days after the first one. This animal developed a small focus of hyalinization. After this observation a series of rabbits (Group VII) was sensitized to human plasma taken from the same person and injected at intervals of 0, 30, and 45 days. The animals were killed after the last injection. A hyaline layer was observed in three out of seven rabbits, but the extent of the lesion was so small that little importance is attached to this experiment. Moreover, atelectasis was wanting.

In considering the investigations that had shown the hyaline membrane to be composed of fibrin, the possibility suggested itself of producing a fibrin clot inside the alveolus. It is well known that the lung parenchyma contains thromboplastin. Moreover, it is possible to produce an intrapulmonary fibrin clot by the steps used by Quick for determination of prothrombin time. With this in mind, an equal part of 0.22% calcium chloride solution was added to human plasma, as in Quick's technique. Since the mixture coagulates in the syringe in a matter of 5 or 10 minutes, it was prepared immediately before injection. Only 5 ml. of the recalcified plasma was injected, less than any other substance, because the rabbits developed a dyspnea more intense than that following the use of any other of our materials.

Unless factors that escape present knowledge are at fault, it must be concluded that the hyaline layers and hyaline casts formed in the alveoli and bronchioli of these rabbits are made up of fibrin.

As a screening step, eight rabbits (Group III) were given injections of recalcified plasma. All of them developed an alveolar fibrin clot and layer, more constant and more extensive both per group and per individual. After this gratifying result, 15 more rabbits were given injections in order to study the evolution of their lesions up to the sixth day.

At the time of the first observation, 12 hours after injection, the lesion had already developed, reaching its maximum between 24 and 48 hours and beginning to recede at about 72 hours. The deposits are not identical with the membrane of the newborn infant but bear a certain resemblance to it. Fully developed, they consist of a cast and a layer of hyaline material of

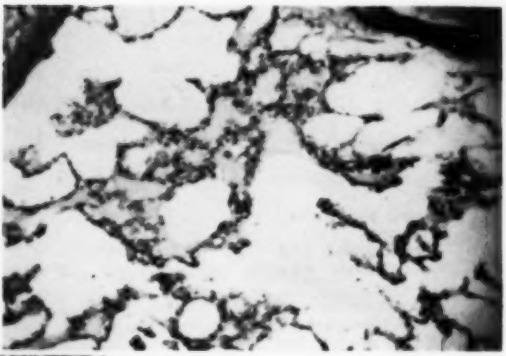

Fig. 1.—Lung alveoli with hyaline casts and layer. No atelectasis is present yet. Rabbit died 10 minutes after injection of recalcified plasma; × 120.

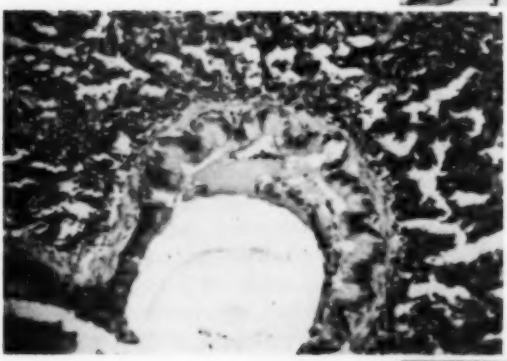

Fig. 2.—Bronchiolus lined with a fibrin layer. Atelectasis around it; × 120.

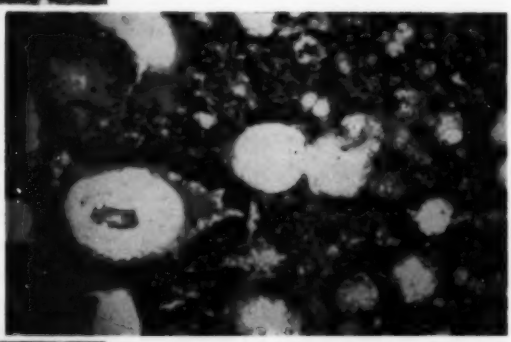

Fig. 3.—Bronchiolus lined with a fibrin layer. Atelectasis is present, and many alveoli are lined by fibrin; × 120.

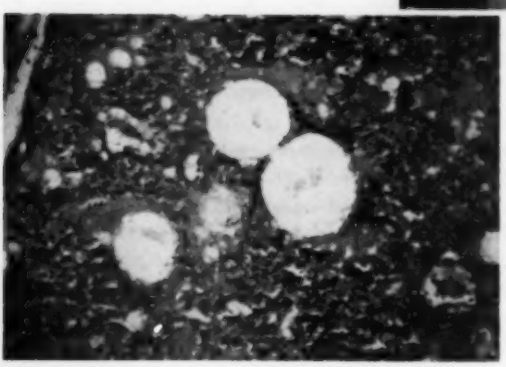

Fig. 4.—Pulmonary alveoli, showing casts and a layer of fibrin. Atelectasis is also seen.

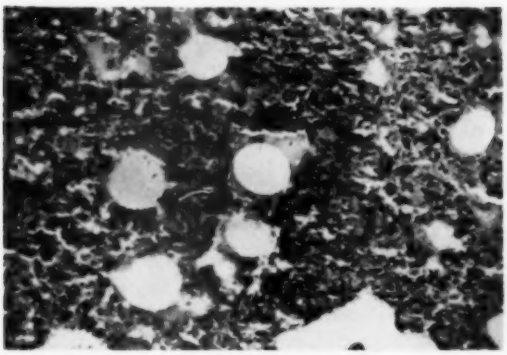

Fig. 5.—Casts and layers of fibrin within pulmonary alveoli.

varying thickness, i. e., a homogeneous, translucent, acellular substance that stains with eosin. The hyaline material in these rabbits is, with little doubt, composed of fibrin. It lines the alveolar and bronchiolar walls; more frequently it fills both structures completely, forming hyaline casts. In all instances the zones with a deposit of fibrin are surrounded by zones of pulmonary atelectasis, the extent of which bears a direct relation to the number of alveoli affected.

The intra-alveolar fibrin casts and layer with atelectasis were found in 100% of the total of 23 animals given injections of recalcified human plasma. Toward the third or fourth day the number of injured alveoli seems slightly decreased. At the same time the fibrin layer begins to show some lysis at its points of contact with the alveolar wall, taking on a festooned appearance. The zone of lysis gradually deepens until the fibrin disappears. Toward the fifth or sixth day the fibrin layer has almost vanished, leaving only scanty alveoli in which a very thin hyaline layer can still be identified. By this time the atelectasis has also disappeared or is at a minimum; in its stead the alveolar walls are moderately thickened, mainly through an agglomeration of intramural macrophages. The capillaries are slightly dilated.

In the course of the first 24 hours the alveolar walls and lumina contain many macrophages; later, on the second day, they also contain a small number of neutrophils and still fewer eosinophils. This cellular infiltrate persists unchanged until the sixth day. It has not been possible to establish a relationship between the amount and the situation of the macrophages and the lysis of the fibrin layer. This lysis occurs on the surface in contact with the alveolar walls, which also contain macrophages.

Summary

A cast and a layer of fibrin has been produced in the pulmonary alveoli of 100% of a series of rabbits given injections intratracheally of a mixture of equal parts of human plasma and 0.22% solution of calcium chloride. This mixture when in contact with the thromboplastin contained in the lung parenchyma forms a clot of fibrin. The region of clotting is surrounded by a zone of pulmonary atelectasis.

The evolution of this fibrin layer was studied for six days following its production. It is well developed after 12 hours, begins to recede toward the third or fourth day, and has practically disappeared toward the sixth day. At this time the atelectasis also disappears.

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Ascaridic Granuloma

An Experimental Study

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Although the nematode Ascaris lumbricoides is of world-wide distribution, the incidence and severity of ascariasis is intimately related to the hygienic level and, therefore, more prevalent in tropical and subtropical countries where substandard living conditions more often obtain. The infection caused is usually a benign self-limited one owing to the short life span of the adult worm (6 to 12 months). In massive and repeated infections a severer clinical picture may develop.

Children infected with Ascaris not infrequently manifest a peculiar syndrome characterized by fever, cough, increased expectoration, eosinophilia, and infiltration of lungs ("Loeffler's syndrome," "tropical eosinophilia"), attributed to the migration of larvae through the pulmonary parenchyma in completing their life cycle in the human host.[1,2] More rarely noted are manifestations of larval migration through the liver.[3-5] Several cases are on record in which Ascaris larvae have been found in "ectopic" locations (optic thalamus,[6] spinal canal,[7] pituitary,[8] epididymis,[9] etc.).

The commonest complications, however, result from heavy parasitization of the intestinal tract. I have observed a case in which over 2000 Ascaris were counted in the intestine of a child. It is not difficult to understand why in such cases episodes of intestinal obstruction, volvulus, gangrene of the bowel, and regurgitation and vomiting of parasites, with aspiration and asphyxia, are occasionally seen. During this retrograde migration, the worms may enter the Eustachian tube to lodge in the inner ear or the mastoid or, by their writhing motion, make their way through the orifices of the paranasal sinuses where they are trapped. Because of the proximity of the parasites in their normal habitat to the orifices of the biliary and pancreatic system, migration into these hollow structures commonly occurs. In extraintestinal locations the worm does not survive long; upon its death abundant parasite proteins are liberated, which, in a patient sensitized to Ascaris antigens, may result in an acute necrotizing inflammation akin to Arthus' phenomenon.[10]

Obstruction of the biliary system has been reported in over 200 instances. Such obstruction may lead to ascending cholangitis, acute cholecystitis,[11-17] or, when the pancreatic duct is entered, to an acute necrotizing pancreatitis.[18] Single or multiple liver abscesses are often observed in ascariasis of the biliary tract.[19-21] It is generally accepted that the suppurative inflammation is due to the luxuriant multiplication of enteric organisms carried in by the worm; abscesses may rupture through the liver capsule, producing a subdiaphragmatic abscess; into the pleural cavity, with development of an empyema, or, more rarely, through the anterior abdominal wall or into the peritoneal cavity. Histologic examination reveals irregular foci of liver necrosis infiltrated by eosinophils and neutrophils, and containing eggs and fragments of the adult Ascaris worm; these abscesses are surrounded by a broad zone of proliferating fibroblasts, foreign-body giant cells, lymphocytes, and monocytes. Adult Ascaries are usually seen

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in the bile ducts at surgery or during necropsy; occasionally they can be demonstrated by radiologic examination with contrast dyes.

There has been a number of cases reported in which the clinical and pathologic features differed from those described above. Monserrat and Africa [22] reported the case of a 3-year-old Filipino, dying as a result of tuberculous meningitis, who, at autopsy, showed a hard round well-circumscribed mass in the portal region; histologic examination revealed numerous Ascaris eggs in different stages of segmentation surrounded by necrotic liver cells and abundant fibrinopurulent exudate. At the periphery there was a large number of foreign-body giant cells and active fibroblastic proliferation, with infiltration by eosinophils and lymphocytes. Neither in the lesion nor anywhere else in the body were adult A. lumbricoides identified. Starkus [23] and Rabinavičius [24] simultaneously reported the case of a 37-year-old woman complaining of intermittent episodes of right upper quadrant pain and jaundice of several years' duration. Because the clinical symptoms suggested a diagnosis of chronic cholecystitis and cholelithiasis, the patient was operated upon. At surgery the gallbladder was normal; however, there were numerous hard round reddish-gray nodules, varying from approximately 1 cm. to 4 cm. in diameter, scattered throughout the upper surface of the liver. As the nodules suggested a metastatic malignant tumor, one was resected for histologic examination. This revealed a central cavity containing necrotic cells, abundant eosinophilic granulocytes, occasional lymphocytes, and numerous Ascaris eggs; at the periphery, there was marked foreign-body giant-cell reaction and fibroblastic proliferation. Adult Ascaris were not identified in the lesion, bile ducts, or intestinal tract. More recently Correa Henao [25] reported on six cases showing symptoms of hepatic disease which, on examination of biopsy material, revealed liver abscesses containing Ascaris eggs without demonstrable remnants of adult parasites. Carrera has also observed a case in which the biopsy specimen obtained from a liver abscess showed abundant Ascaris eggs.[26] Matsubayashi [27] states that the finding of Ascaris eggs in routine autopsy material is not uncommon in Japan, even in the absence of adult parasites in the biliary or intestinal tracts.

It is now well established that Ascaris worms may transitorily migrate into the biliary system and reenter the intestinal cavity. An adult female Ascaris can lay from 200,000 to 250,000 eggs per day; it is possible that when lodged in the bile ducts the hostile ambiance may increase the parasite activity and accelerate the discharge of eggs. During this brief sojourn, enough eggs may be deposited to result in severe inflammation.

There is, however, inadequate evidence as to the role played by Ascaris eggs alone in the induction of such inflammatory reaction. Monserrat and Africa [22] suggested that the egg, besides acting as a foreign body, must secrete some toxic substance to which a great part of the necrosis and inflammation is due. Although Correa Henao [25] produced liver abscesses in guinea pigs, by injection of suspensions of Ascaris eggs, the lesions induced were examined three weeks after the inoculation of the suspension of eggs. This leads the author to believe that the inflammatory reaction is a typical foreign body granuloma; he does not mention the possible role of toxins produced by the eggs as a factor in the causation of the inflammation.

In reviewing the literature I have been unable to find a detailed experimental study as regards the evolution of the inflammatory response resulting from the presence of Ascaris eggs. Moreover, no effort has been made to exclude the possible role played by other pathogenic organisms in the induction of such an inflammatory reaction. The following experiments were, therefore, carried out in order to investigate the histopathologic development of these lesions in the absence of pathogenic organisms other than Ascaris eggs.

Materials and Methods

A. lumbricoides eggs were obtained by stripping the uteri of adult female worms. The eggs were washed repeatedly in sterile saline solution and then placed in 2% formalin for 24 to 48 hours. Prior to injection, the eggs were again repeatedly washed in sterile saline solution, and suspensions containing from 16,000 to 32,000 eggs per milliliter of suspension were made.

Albino rabbits weighing an average of 2500 gm. were utilized throughout these experiments. The animals were anesthetized with ether or pentobarbital (Nembutal) and laparotomized under aseptic conditions. Ascaris egg suspension was then injected as follows: 1. Ten rabbits were given 3 ml. of egg suspension (containing approximately 16,000 eggs per milliliter) into the portal vein. 2. Ten rabbits were given injections of 3 ml. of egg suspension (containing from 16,000 to 32,000 eggs per milliliter), in portions of 0.5 ml., into six different regions of the liver parenchyma. 3. Another group of 10 rabbits were simultaneously given injections of 1.5 ml. of egg suspension (32,000 eggs per milliliter) into the portal vein and 1.5 ml., distributed in three equal parts, into distant regions of the liver parenchyma. The animals were sutured and returned to their cages. Six additional rabbits obtained from the same source, but not given injections of egg suspension, served as controls. The animals were killed at the following intervals: 2, 4, 8, 16, and 36 hours and on the 4th, 8th, 15th, 30th, and 60th day after injection of the suspension. The tissues were fixed in Zenker-formol and in 10% neutral formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Results

The severity of the lesions varied in proportion to the number of eggs present at a particular site. After the egg suspension was injected into the portal vein, the eggs became lodged in the small radicles of this vessel. The alterations resulting from the presence of single eggs were as follows: Two hours after injection, the eggs appeared surrounded by a continuous layer of eosinophilic granulocytes (Fig. 1). This occurred around both intact and partly degenerated eggs. The sinusoids of the surrounding liver contained large numbers of eosinophils, which occasionally infiltrated the periportal connective tissue. The eosinophilic infiltrate increased markedly during the following six hours; at this time focal degeneration of the hepatocytes at the periphery of liver lobules in contact with the lesion was also noted (Fig. 2). Mononuclear cells began to appear in the exudate 8 hours after the injection of egg suspension and constituted a large part of the inflammatory cells in lesions older than 16 hours. Although accumulation of small hyperchromatic nuclei, suggesting early formation of multinucleated giant cells, became apparent in 8-hour-old lesions, the characteristic foreign-body giant cell did not ap-

Fig. 1.—Two-hour-old lesions. Ascaris egg surrounded by eosinophilic granulocytes in a portal radicle. Note the presence of eosinophils in adjacent sinusoids of liver. Hematoxylin and eosin; × 430.

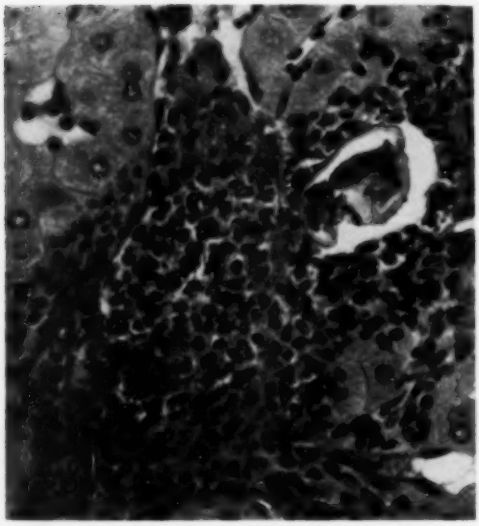
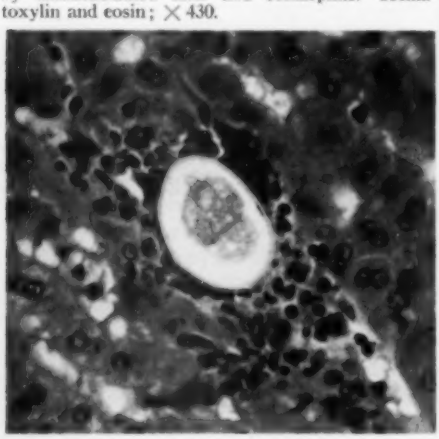


Fig. 2.—Eight-hour-old lesion. Partly degenerated Ascaris egg in lumen of portal radicle. Severe eosinophilic infiltrate in periportal space; increasing numbers of monocytes. Hematoxylin and eosin; ×430.

pear until the lesions were 16 hours old or older (Fig. 3). In 36-hour-old lesions, in addition to foreign-body giant-cell reaction and intense eosinophilic infiltrate, proliferation of fibroblasts was also beginning (Fig. 4). At this stage most eggs were degenerated, and occasionally only remnants of the chitinous shell could be identified in the cytoplasm of giant cells and other macrophages (Fig. 5). Four days after injection, the lesions were composed of abundant eosinophils, monocytes, histiocytes, proliferating fibroblasts, and foreign-body giant cells (Fig. 6). Pseudotubercle formation with abundant epithelioid cells and more intense fibroblastic proliferation were characteristic findings in seven-day-old lesions (Fig. 7). Two weeks after injection, the granulomas were surrounded by broad bands of connective tissue fibers, infiltrated with sparse numbers of eosinophils and lymphocytes (Fig. 8). Increased amount of fibrous connective tissue was seen along the periportal spaces extending between adjacent lobules. This change was more prominent in four-week-old lesions (Fig. 9). At this time there were partial hyalinization of the connective tissue and scattered pseudotubercles, some still containing fairly well-preserved Ascaris eggs. In other regions the lesions appeared as pseudotubercles without any remnants of the egg (Fig. 10); these granulomas showed a central portion made of large numbers of epithelioid cells, surrounded by concentric bands of connective tissue fibers infiltrated with eosinophils, lymphocytes, histiocytes, and occasional giant cells. Only rarely was fibrinoid degeneration of the central portion of these tubercles noted (Fig. 11). Animals killed two months after initiation of the experi-

Fig. 3.—Sixteen-hour-old lesion. Ascaris egg in 2-cell stage of blastomeric division, surrounded by multinucleated cells and eosinophils. Hematoxylin and eosin; ×430.



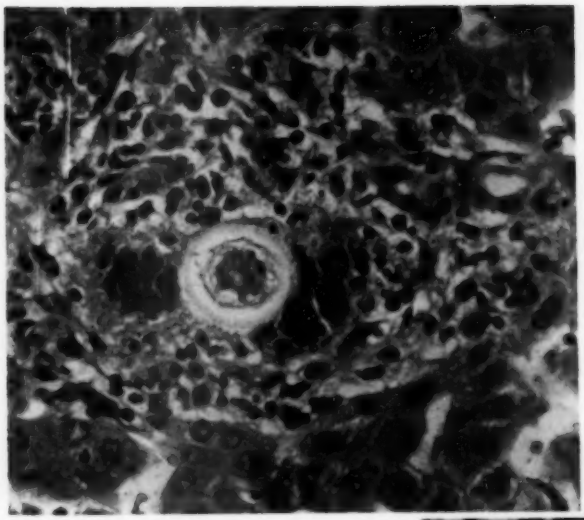


Fig. 4. — Thirty-six-hour-old lesion. Note granulomatous inflammation with giant cells containing degenerated eggs. Hematoxylin and eosin; × 430.

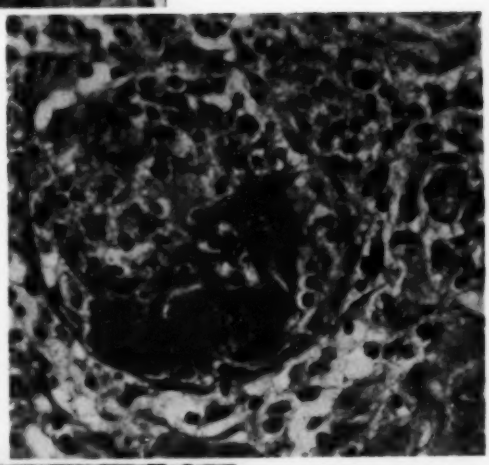


Fig. 5.—Thirty-six-hour-old lesion. Granulomatous inflammation with giant cells containing fragments of degenerated Ascaris eggs; marked eosinophilic infiltrate. Early fibroblastic proliferation. Hematoxylin and eosin; × 430.

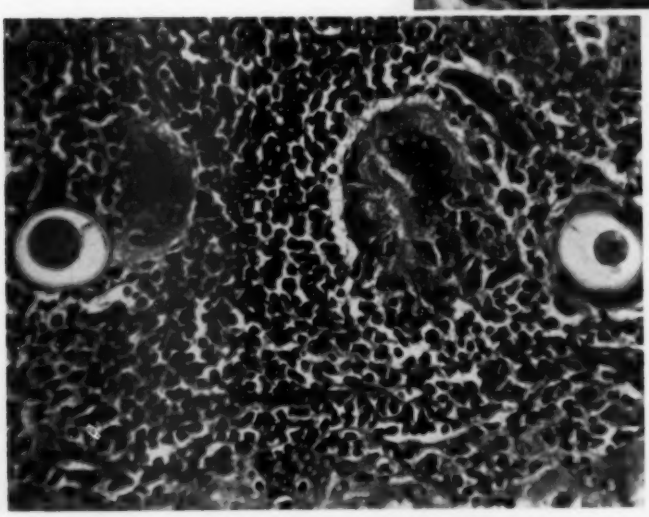


Fig. 6.—Four-day-old lesion. Large area of granulomatous inflammation in portal space. Eggs are well preserved and are being surrounded and engulfed by giant cells. Monocytes and eosinophils are abundant. Hematoxylin and eosin; reduce slightly from mag. × 215.

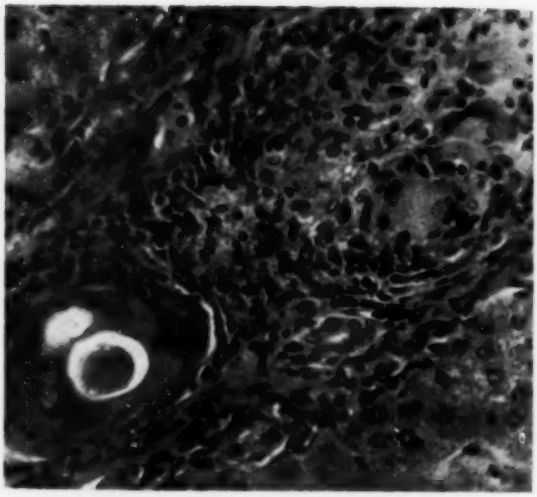


Fig. 7.—Seven-day-old lesion. Degenerated Ascaris eggs in cytoplasm of giant cells. Note pseudotubercle formation at upper right-hand side of picture. Hematoxylin and eosin; × 215.

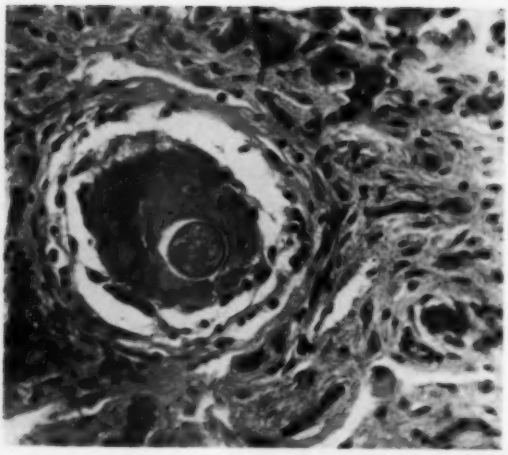


Fig. 8.—Two-week-old lesion. Intense fibrosis of periportal space about a granuloma. Note fairly well-preserved Ascaris egg within cytoplasm of giant cell. Hematoxylin and eosin; × 215.

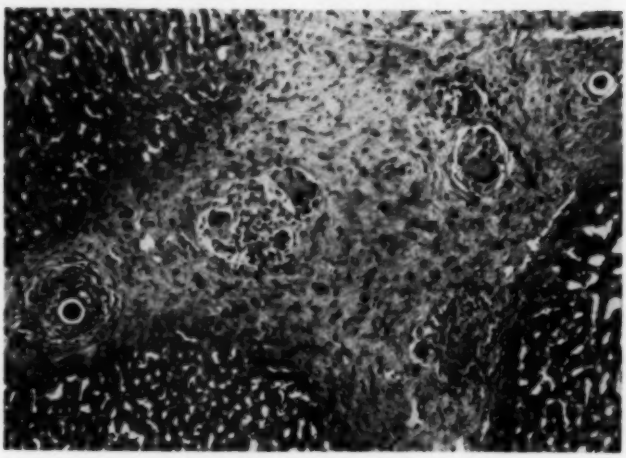


Fig. 9.—Four-week-old lesion. Extensive fibrosis and broadening of portal spaces; scattered pseudotubercles, some of which contain intact eggs. Hematoxylin and eosin; × 100.

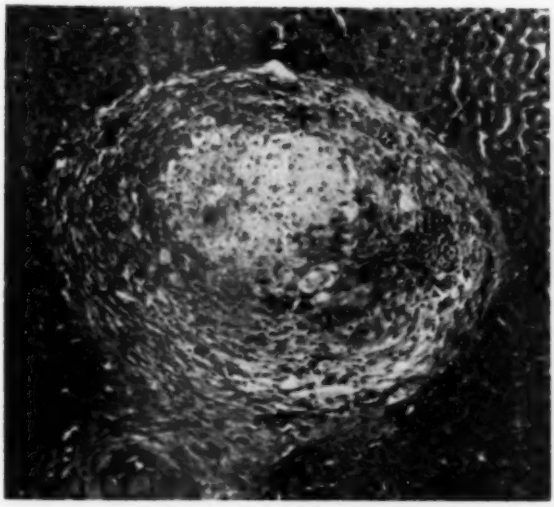


Fig. 10.—Five-week-old lesion. Typical pseudotubercle; note accumulation of epithelioid cells in center and intense fibroblastic proliferation at the periphery. Moderate infiltration by eosinophils and lymphocytes. Hematoxylin and eosin; × 100.

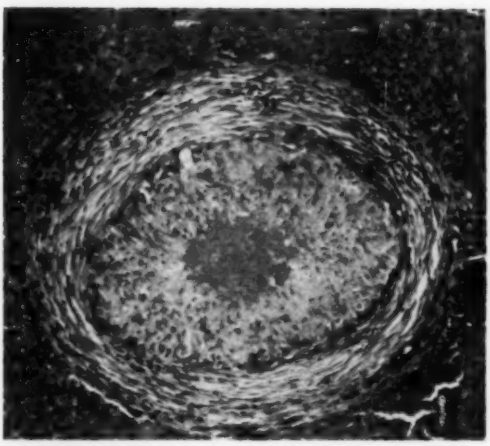


Fig. 11.—Five-week-old lesion. Pseudotubercle with central fibrinoid necrosis. Hematoxylin and eosin; × 100.

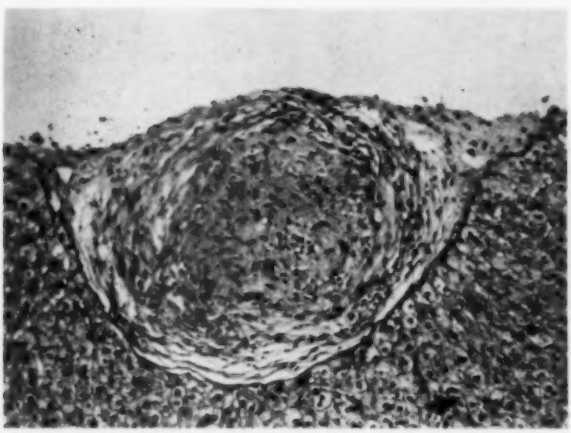


Fig. 12.—Two-month-old lesion. Partly hyalinized pseudotubercle, resulting from Ascaris egg injection. Hematoxylin and eosin; × 100.

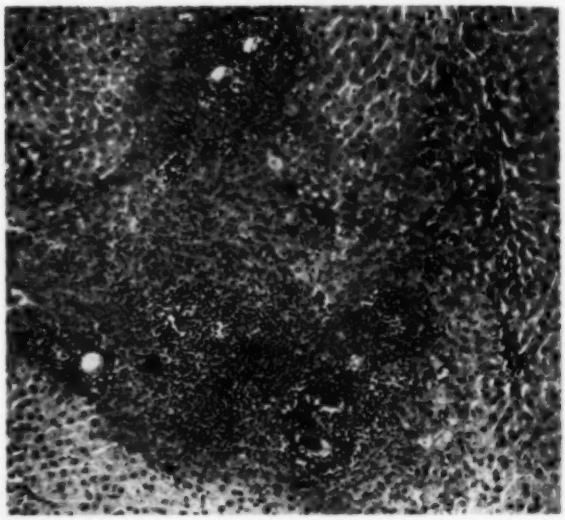


Fig. 13.—Four-hour-old lesion, after injection of Ascaris eggs in liver parenchyma. Compare the severity of the inflammatory response with that in Figure 2. The large majority of cells are eosinophils and red blood cells. Necrotic hepatocytes are also present. Hematoxylin and eosin; × 100.

ments revealed, for the most part, complete hyalinization of the pseudotubercles.

Lesions induced after injection of larger concentrations of egg into the liver parenchyma differed from those described above only in the severity of the inflammation and in the residual scarring (Figs. 13 through 16). In 16-hour-old lesions, large abscesses were noted which, when located at the periphery of the liver, often resulted in rather extensive adhesions to the omentum or diaphragm. In late stages focal calcification of the granulomas was prominent (Fig. 17).

Most of the injected eggs were digested or were in various stages of disintegration within 16 hours after initiation of the experiment. However, others were still unaffected, and blastomeric division proceeded unhampered, even in eggs located within the cytoplasm of giant cells (Figs. 3 and 15). Intact eggs were also found in giant cells

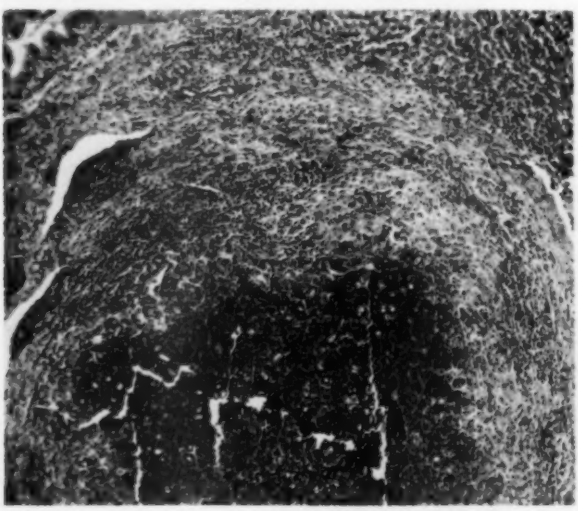


Fig. 14.—Sixteen-hour-old lesion. Note the large size of the abscess, which contains abundant Ascaris eggs and eosinophils. There is extensive necrosis of hepatocytes, moderate numbers of epithelioid cells and fibroblasts. Hematoxylin and eosin; × 50.

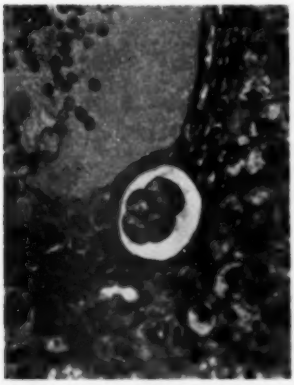


Fig. 15.—Seven-day-old lesion. Note pseudotubercle formation. A well-preserved Ascaris egg is in the four-cell stage of division, on the right upper portion of the picture. Hematoxylin and eosin; × 215.

in 2-month-old lesions. Blastomeric division was never observed past the eight-cell stage.

Comment

It is obvious from these experiments that the injection of Ascaris eggs into rabbits causes an inflammatory response, the severity of which is directly proportional to the number of eggs present at a particular site and that neither bacteria nor products resulting from disintegration of adult parasites are necessary for the induction of such response.

Ascaris eggs show a remarkable eosinophilotactism which becomes apparent within two hours after injection and increases in severity during the following six hours. The

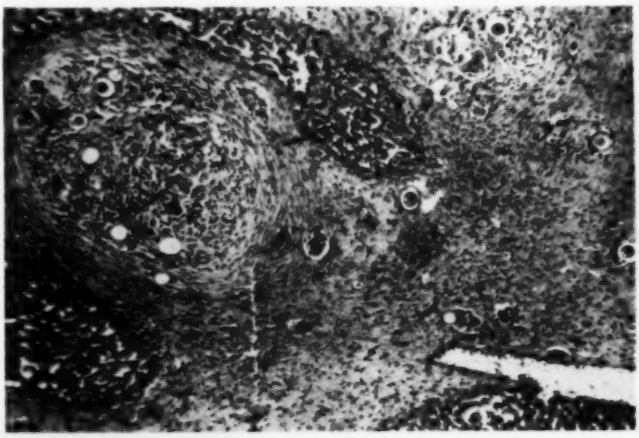


Fig. 16.—Four-week-old lesion. Extensive fibrosis replacing an area of previous abscessification. There are still many viable eggs, some of which are in the eight-cell stage of blastomeric division. Hematoxylin and eosin; × 215.

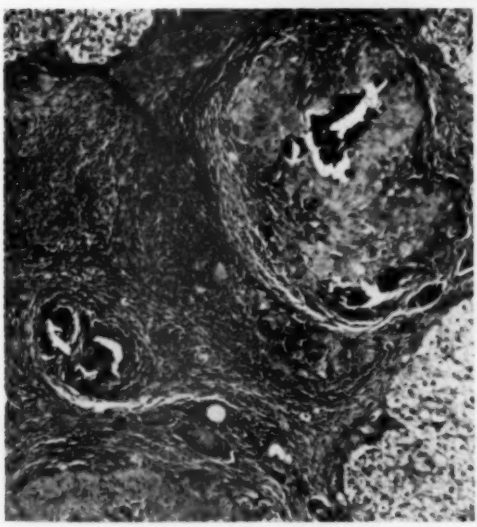


Fig. 17.—Four-week-old lesion. Focal areas of calcification in the center of pseudotubercles. Hematoxylin and eosin; × 60.

mechanism by which this outpouring of eosinophils occurs has not been elucidated; there is little doubt, however, that either some product of the metabolism of the egg or a chemical constituent of the egg itself must play an important and decisive role in the development of the lesions. The similarity of the inflammatory reaction about intact eggs (as evidenced by their ability to proceed with blastomeric division) as compared with that found around disintegrating eggs suggests that the toxic substance is very likely a component of the egg shell. Campbell [28] has shown that keratin obtained from adult Ascaris also causes a pronounced eosinophilia; this, however, is not noticeable until several days after the injection. Campbell points out that eosinophilotactism is not due to the antigen per se but results from its insolubility, which allows it to remain in the animal body after specific antibodies are formed, thus giving rise to a hypersensitivity reaction. The rapidity with which eosinophilic infiltrates occur in the lesions herewith described makes it safe to rule out hypersensitivity as an explanation, at least during the early phases of the inflammatory reaction.

Also striking is the appearance of multinucleated giant cells within a few hours after injection of Ascaris eggs (Figs. 3-5). Although more numerous in areas where degeneration of eggs has occurred, giant cells are also seen surrounding eggs with intact shells and viable embryos. It is very likely that the substance responsible for this granulomatous change is a component of the outermost or of the chitin layers; the possibility that phosphatides or other lipids from the embryo may seep out into the tissues to induce transformation of monocytes into giant cells cannot be excluded.

Most lesions proceed to heal rapidly by hyalinization, the process taking place between the fourth and the eighth weeks. In some instances, however, a number of pseudotubercles remain in which changes indicative of an allergic-hyperergic phenomenon are noted; these are characterized by swelling of epithelioid cells leading to extensive fibrinoid necrosis and disappearance of the eggs as identifiable structures (Fig. 11). Worthy of mention is the fact that such findings are encountered only in lesions 4 weeks old or older, at a time when antibodies against the eggs are readily demonstrable. Mercer et al.,[5] Perlingiero and György,[3] von Meyenburg,[2] and others have described similar lesions in the liver of children suffering from Ascaris infections. Interestingly enough, Ascaris larvae were found only in early granulomatous lesions but not in others showing fibrinoid necrosis. Zuelzer and Apt [29] have reported eight cases in which a clinical picture characterized by hepatomegaly, pulmonary infiltration, asthmatic complaints, hyperglobulinemia, and pronounced eosinophilia in bone marrow and peripheral blood was associated with the presence of granulomas with focal necrosis and widespread eosinophilic infiltrates in sections of liver obtained by biopsy or at autopsy; he was unable to demonstrate the presence of ascariasis in the lesions by skin sensitivity tests. Since it is known how rapidly Ascaris larvae can be destroyed in hypersensitive persons, their absence in the lesions is not proof against their being the responsible agent. Moreover, negative skin tests do not necessarily reflect lack of infection but may be due to an anergic state. Although other etiologic agents may be responsible for a similar clinical and histopathologic feature, before A. lumbricoides is ruled out as an etiologic factor, more thorough serologic testing should be made.

The capability that Ascaris shows for migration into the biliary and pancreatic system and also through the intestinal wall makes it possible for it to release abundant eggs into these cavities or tissues, resulting in abscess formation or granulomatous inflammation. If the eggs are released in the peritoneal cavity, they may be trapped in a single fashion and result in scattered pseudotubercles. Unless these granulomas are examined prior to complete disintegration of the egg, they may be confused with those caused by other organisms (Mycobacterium tuberculosis, fungi) with the consequent

prognostic and therapeutic implications. It is also well to remember that A. lumbricoides is second only to Endamoeba histolytica among parasites causing abscesses of the liver.

Summary

Experimental inoculation of Ascaris lumbricoides eggs into the liver of rabbits results in an inflammatory reaction, the severity of which is proportional to the size of the inoculum. The lesions were induced in the absence of bacteria or products of the adult parasite.

The characteristic cells of the early stages of inflammation are eosinophils. These are followed within 16 hours by increasing numbers of monocytes, giant cells, and fibroblasts. Pseudotubercle formation becomes apparent about the end of the first week after inoculation.

Healing takes place by hyalinization with more or less extensive fibrosis, depending on the number of eggs present at the particular site. In lesions resulting from large inoculums, abscess formation is apparent within 16 hours after the inoculation.

The similarity of the lesions induced experimentally to those reported in human infections is discussed.

Dr. J. Oliver-Gonzalez, Professor of Parasitology, University of Puerto Rico, supplied Ascaris eggs for these experiments, and Mr. Agustin Fernandez helped during the operative procedures.

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The Effect of Estrogen on Thyroid Structure and Function

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An intimate relationship between goiter of all types and femaleness has long been recognized. A satisfactory explanation of this relationship has been wanting, and the numerous investigations to elucidate the problem have been conflicting and inconclusive. The effect of estrogen on thyroid structure and function in several animal species has been variously described as involutionary, stimulatory, or nil (bibliography of References 1, 2, and 3). For the rat, the consensus has been that estrogen caused histologic evidence of decreased activity or involution.[4-12] On the other hand, recent studies have shown that several measurable functions of the thyroid were enhanced by the administration of estrogen.[1-3,13,14] An investigation was therefore initiated to correlate both the structural and the functional changes in the thyroid elicited by estrogen and to explain, if possible, the relationship of goiter to the ovarian hormone.

Under the influence of estrogen, a dichotomy of structure and function was observed in the hyperplastic iodine-deficient thyroid. This was not found to occur in the thyroid receiving an adequate supply of iodine.

Materials and Methods

Castrate male rats, operated on 10-12 days before each experiment, were used. Three experiments were done.

A. Thirteen rats were divided into two groups, six experimental and seven control. The experimental animals received subcutaneously daily for five days 2000 I. U. of estrogen* (200μg. of estrone) in 0.2 ml. of saline for a total of 10,000 I. U. (1.0 mg.). Controls received no injections. All animals were maintained with tap water and a diet of Lab Blox† containing adequate iodine as potassium iodide.

B. Fourteen rats were divided into two groups, of seven experimental and seven control. The experimental animals received subcutaneously daily 100 I. U. of estrogen (10μg. of estrone) in 0.2 ml. of saline for eight weeks; the total dose was 5500 I. U., or 0.55 mg. Controls received no injections. They were all maintained on doubly distilled water and an iodine-deficient diet‡ to which had been added 20μg. of iodine (as NaI) per 100 gm. of diet.

C. Fourteen rats were divided into two groups, of seven experimental and seven control. The experimental animals received subcutaneously daily 100 I. U. of estrogen (10μg. of estrone) in 0.2 ml. of saline every two days three times weekly for seven weeks; the total dose was 2000 I. U., or 0.20 mg. Controls received no injections. They were maintained with doubly distilled water and an iodine-deficient diet (as in Group B) which was effectively goitrogenic.

Twenty-four hours before autopsy the rats of the three groups were given injections of 5μc.-6μc. of I^{131}§ intraperitoneally. At autopsy the thyroids were immediately dissected free of adventitial tissue, weighed to the nearest 0.1 mg. on a torsion balance, and placed in fixative for serial sectioning and periodic acid-Schiff (PAS) staining. The fixed glands were counted in a well type thallium-activated NaI crystal counter with a sensitivity of 1×10^6 cpm per microcurie of I^{131}.

The height of follicular epithelium was measured with an ocular micrometer. Fifty measure-

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* Theelin—Parke, Davis & Company, 5 mg. of estrone per cubic centimeter.

† Wayne Lab-Blox, 0.00043% KI.

‡ Nutritional Biochemicals Corp., Clevelend 28. The diet contained approximately 1μg. % of iodine.

§ Obtained from Oak Ridge National Laboratories, Oak Ridge, Tenn.

Effect of Estrogen on Thyroid Structure and Function

Group		Rats, No.	Body Wt., Gm.	Thyroid Wt., Mg.	Thyroid Wt., Mg. % B. W.	Uptake of I^{131} 100 Mg. of Thyroid, % of Dose	Height of Follicular Epithelium, μ
A	Controls	7	315± 8.4 *	13.0±0.40	4.0±0.20	90.1± 7.7	12.1±0.55
	Estrogen (1.0 mg. over 5 days)	6	308±10.7	14.3±0.32 †	4.6±0.14 †	152.7±10.7 ‡	12.0±0.49
B	Controls	7	353±12.6	14.4±0.34	4.2±0.15	34.8± 2.13	10.6±0.34
	Estrogen (0.55 mg. over 8 wk.)	7	286± 7.6	13.9±0.50	4.9±0.14	45.4±2.31	9.9±0.41
C	Controls	7	363± 9.2	41.3±1.85	11.4±0.42	226.8±24.5	13.2±0.72
	Estrogen (0.20 mg. over 7 wk.)	7	271±10.8	32.5±1.09	12.0±0.59	344.3±16.1	9.9±0.68

* ± standard error of the mean.
† Significantly different from controls at 5% - 2% level.
‡ Underlined figures are significantly different from the control value at the 1% level or less.

ments were taken in each gland, from cells clearly delineated at their basal and luminal borders. An equal number of peripheral and central follicles, transversely cut, was studied.

Results

A. In the Table, Part A, the results of the first experiment are shown. Under the influence of relatively large doses of estrogen, for five days, the thyroidal uptake of I^{131} was markedly increased, compared with control uptake. Despite this functional difference, no structural change was noted in the estrogen-treated thyroids. There were no microscopical alterations to account for the somewhat increased absolute and relative thyroid weights of the experimental group compared with the control group. The one anatomical measurement taken, follicular cell height, was the same in both groups. Though there was some histologic variation (size of follicles, quantity of colloid, staining of colloid, vascular channels, etc.) among the animals of the two groups, it was not constant or greater than the variation within groups.

B. Since no morphologic alteration was induced by the brief administration of large doses of estrogen over a short period, it was thought that the more prolonged administration of the ovarian hormone might accomplish this. Smaller doses were used because large doses of estrogen over eight weeks debilitated the rats.

In the Table, Part B, the data of the second experiment are shown. As above, the administration of estrogen for eight weeks augmented thyroidal accumulation of I^{131} but did not alter thyroidal structure. The absolute thyroid weight was not significantly different in the two groups of rats. However, owing to the marked weight loss in the animals receiving estrogen, the relative thyroid weight was significantly greater in the experimental group as compared with the control. Follicular epithelial height and over-all histology were unaffected by the hormone.

C. It was apparent that although estrogen influenced thyroidal accumulation of I^{131} it did not alter thyroidal structure in rats receiving adequate iodine in their diet. It might be possible, however, to affect thyroid structure if the gland were hyperactive, i. e., a morphologic effect might be more easily visualized if the thyroid were deficient in iodine.

In the third experiment, Part C of the Table, the administration of estrogen in relatively small doses for a period of seven weeks elicited both functional and structural changes (Figs. 1 and 2). Under the influence of estrogen, thyroidal accumulation of I^{131} was augmented, absolute thyroid weight was significantly depressed below control levels, and follicular epithelium was significantly lower than that of controls. Histologic examination revealed also, in

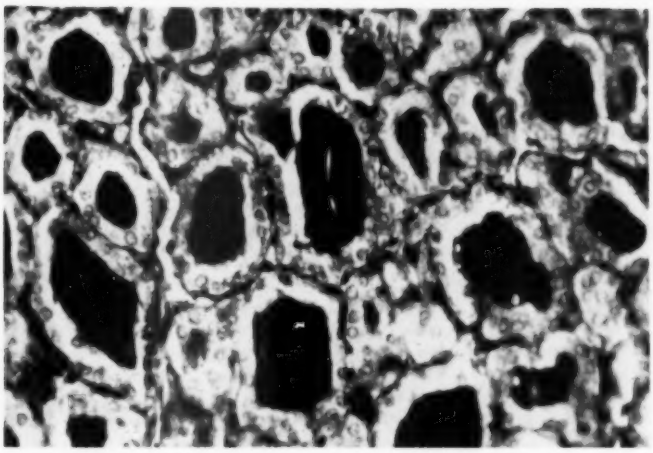

Fig. 1.—Involutionary changes in thyroid of rat given iodine-deficient diet and estrogen. Follicular epithelium is cuboidal; follicles and follicular lumens are larger, and colloid is increased, compared with Figure 2. Reduced 15% from mag. × 240.

estrogen-treated thyroids, an increased quantity of colloid, an increased depth of colloid staining, and an enlargement of follicular lumens, all features of involution (Fig. 1). In comparison, control thyroids showed high columnar epithelium, decreased follicular size, and decreased colloid content and staining (Fig. 2). As before, the experimental animals weighed significantly less than their respective controls.

Comment

Estrogen elicited in rats given an iodine-deficient diet all the characteristics of thyroid involution; flattening of epithelium, increased colloid content, enlargement of follicles, etc. Despite the involutional changes, at least one measured activity, thyroidal uptake of I^{131}, was significantly stimulated. This was an interesting dichotomy of structure and function, precisely the opposite of what is found during the administration of antithyroid drugs, i. e., morphologic hyperplasia and hypertrophy associated with hypofunction. On the other hand, in rats receiving adequate iodine in their diet, estrogen did not alter thyroid morphology, while thyroidal uptake of I^{131} was still enhanced. I have no reasonable explanation for these results at the present time. Others have reported that estrogen caused atrophy or involution in the thyroids

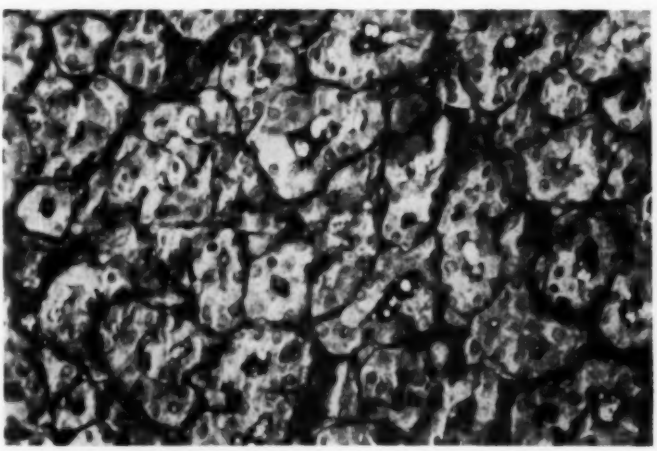

Fig. 2.—Hyperplastic thyroid of rat given iodine-deficient diet. Follicles are small; epithelium is columnar, and colloid and follicular lumens are reduced. Reduced 15% from mag. × 240.

of rats presumably ingesting adequate iodine.[4-12]

It was apparent that under the special conditions of deficient iodine intake estrogen not only did not produce goiter but actually accomplished the opposite. Similarly, this ovarian hormone caused a decrease in thyroid weight when the gland was made hyperplastic and iodine deficient by the administration of propylthiouracil.[10,12,15] It appeared, therefore, that whatever the causal connection between femaleness and goiter, estrogen was not necessarily the etiologic factor. Since estrogen, however, was shown to affect a number of parameters of thyroidal metabolism, usually indicating stimulation, perhaps much longer periods of hormonal administration would be needed to induce goiter. The results of this latter investigation have been reported elsewhere.[25]

The locus of action of estrogen on the thyroid was not determined in these experiments. The hyperplasia and hypertrophy that develop with deficient iodine intake have been attributed to increased TSH production and discharge from the pituitary. Hence, the involutional and atrophic changes induced by estrogen in the iodine-deficient thyroid might be ascribed to inhibition of hypophyseal TSH at its source (inhibition of production, of discharge from the pituitary, or of both) or its peripheral utilization. There is no direct evidence bearing on this matter. Several reports have shown that estrogen diminished hypophyseal content of TSH,[16,17] which is quite different from altering hypophyseal production and discharge of TSH. Others have interpreted their observations to indicate that estrogen stimulated or inhibited pituitary TSH.[18-24] The fact that estrogen directly affected the thyroid, without the intervention of the pituitary, provided an alternative explanation for these results.[2,3] At present, then, the action of estrogen on thyroid function and structure is still incompletely known, and its relationship to the pathogenesis of goiter remains unclear.

University of Pittsburgh School of Medicine (13).

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The Effect of Chemotherapeutic Agents on the Growth and "Differentiation" of Bacteria

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It has been demonstrated that certain antimitotic agents can stop the division of cells without affecting their differentiation. In the case of the intestinal epithelium of rats, for example, the synthesis of mucus and the resultant differentiation of reserve cells into goblet cells continues despite doses of irradiation, mustards, or aminopterin which inhibit division.[2] Similarly, the maturation of sperm in the seminiferous epithelium of rats and mice proceeds after treatment with doses of mechlorethamine hydrochloride (nitrogen mustard) or irradiation which interfere with the mitosis of the more undifferentiated elements.[3] In experimental tumors in mice, as in spontaneous tumors in man, one of the characteristic effects of ionizing irradiation or certain alkylating agents is increase in the cytoplasmic mass of the treated cell due to persistent protein synthesis and a concomitant increased formation of specific cytoplasmic products despite failure of division. For example, poorly keratinizing or transitional epidermoid carcinomas produce more keratin and melanomatous cells become loaded with pigment after irradiation.[4]

Conversely, certain other antimitotic agents inhibit both differentiation and division. Urethane and colchicine, for example, interfere with the differentiation of goblet cells in the jejunal mucosa[2] and do not permit the maturation of sperm,[3] while colchicine affects the production of pigment in experimental melanomas.[4]

The suggestion has been made that the effects of the antimitotic agents which inhibit differentiation are mediated through interference with RNA activity and that the locus of action of the modalities which permit differentiation but inhibit division is DNA.[4] In order to obtain material more suitable for chemical analysis than the tumors and tissues under study, it was decided to employ bacteria.

One of the well-known effects of antibacterial agents, growth inhibitors, and other substances on bacteria is the production of giant and filamentous forms. They are, of course, also seen in aging cultures and under a variety of unfavorable nutritional and environmental conditions. These forms seemed somewhat comparable to the giant cells produced by chemotherapeutic agents and irradiation in mammalian cells. Preliminary surveys were made of the effects of irradiation, mechlorethamine hydrochloride, colchicine, and urethan on the morphology and chemical reactions of strains of Pseudomonas, paracolon species, Proteus, and Candida by the addition of the chemical agents and P^{32} to the cultures. Giant forms were readily produced (Figs. 1 and 2). It was furthermore observed that urethan inhibited pigment formation by Pseudomonas at a concentration one-tenth of that required to produce giant cells, which phenomenon was generally associated with the beginning inhibition of growth. A concentration one-fifth of that necessary to inhibit growth of the culture completely suppressed pigment formation. It was

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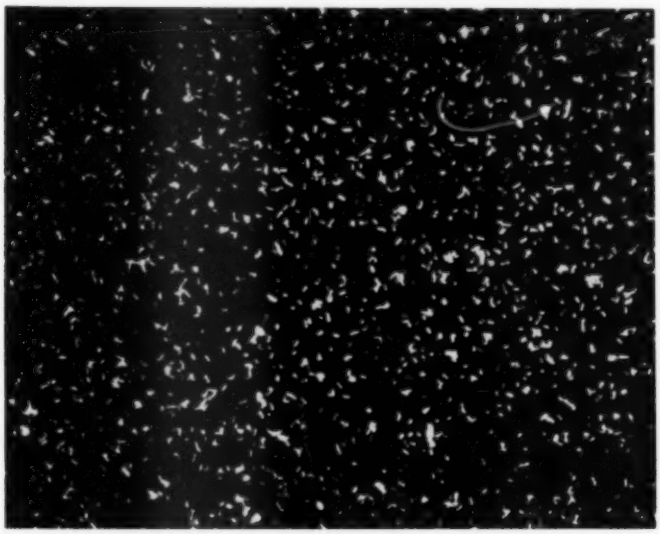


Fig. 1. — Photomicrograph of smear from control culture of Pseudomonas. Nigrosin; reduced about 10% from mag. × 1200.

therefore decided to study this phenomenon in greater detail.

Methods

Strains of Pseudomonas aeruginosa were grown on slants of nutrient agar and buffered casein hydrolysate agar. Solutions of urethan and mechlorethamine hydrochloride were added to the culture medium in varying amounts, and the final volumes employed for the slants were equalized. The range of the resulting concentrations were from 0 to 15 mg. per milliliter of urethan and 0 to 1 mg. per milliliter of mechlorethamine hydrochloride. The cultures, incubated at 37 C, were studied at 12, 18, 24, and 48 hours. Some were also studied at 8, 36, and 72 hours.

The growth on the surface of the slant was collected in 5 ml. of distilled water. A 0.1 ml. aliquot was diluted in a Coleman cuvette until it gave a reading between 30 and 100 Coleman nephelos units. Nephelos units were calculated according to the

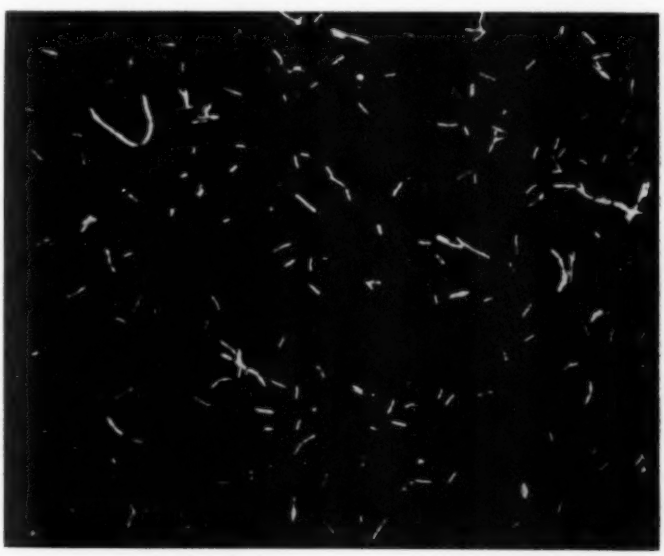


Fig. 2. — Photomicrograph of smear from mustard treated culture of Pseudomas. Note giant forms. Nigrosin; reduced about 10% from mag. × 1200.

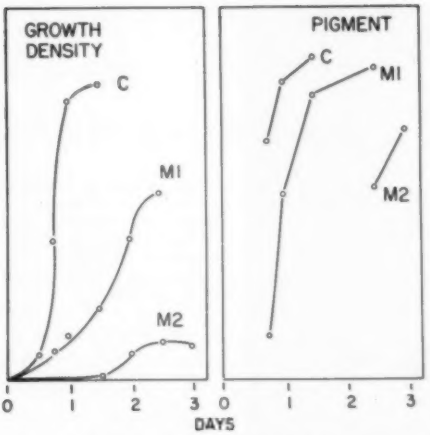


Fig. 3.—Compares pigmentation and growth of Pseudomonas in control cultures (*C*) and in cultures containing mechlorethamine hydrochloride. *M1* is at a concentration of 0.3 mg. per milliliter, and *M2*, of 1.0 mg. per milliliter. The highest control value plotted in this figure is 960 units. The highest control value plotted for pigment is 0.52.

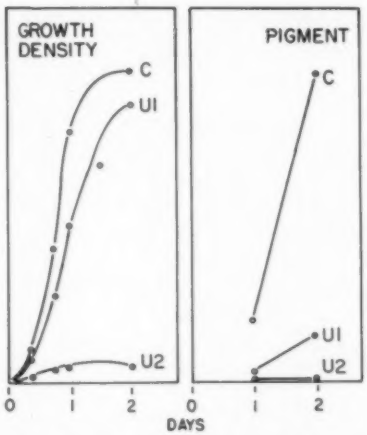


Fig. 4.—Similar to Figure 3, with the exception that urethan was added to the cultures in place of mechlorethamine hydrochloride. The concentration of urethane was 10 mg. per milliliter for *U1* and 15 mg. per milliliter for *U2*. The highest control points plotted are 500 units for growth density and 1.24 for pigment.

linear scale of Coleman nephelos standards as established by the operating instructions for the Coleman Model 9 Nepho-Colorimeter.[5] The growth density was expressed as the nephelos units multiplied by the dilution and divided by 10.

Upon the addition of 5 ml. of chloroform to the culture slant, the pigment was extracted for 18 hours, after which it was filtered and diluted to a total volume of 10 ml. with chloroform. The amount of pigment in the chloroform extract was then estimated colorimetrically in a Coleman Model 9 Nepho-Colorimeter with use of a 655 mμ (red) filter. Pigment concentration was expressed in terms of optical density, which was found to have a linear relationship throughout the various ranges of pigment concentration tested.

Colony counts were also performed and packed cell volumes measured as well, but the most significant results were obtained when the turbidometric density and the intensity of pigment were plotted in parallel (Figs. 3 and 4).

Results

The results are shown graphically in Figures 3 and 4 and visually in Figures 5 and 6.

The inhibition of pigment production by mechlorethamine hydrochloride was proportionately less than the inhibition of growth. Considerable pigment was produced at concentrations of mechlorethamine at which there was significant interference with growth. Conversely, urethan markedly inhibited pigment production at concentrations which interfered very little with growth, and pigment formation could be blocked completely at concentrations which still permitted some growth.

Comment

It is interesting that urethan, which inhibited differentiation in the mammalian cells studied, inhibits the formation of pigment by Pseudomonas, whereas mechlorethamine hydrochloride, which did not inhibit the differentiation of mammalian elements, does not inhibit formation of pigment by Pseudomonas. Incidentally, iodoacetate has been reported as inhibiting formation of pyocyanin but not growth.[8]

If differentiation can be defined[6] as the act or process of acquiring individual characters, one might consider the production of pigment by Pseudomonas to be as indicative of differentiation as the keratinization of an epidermal cell or the pigmentation of a melanomatous element. Since RNA plays a key role in enzyme forming systems[7] and

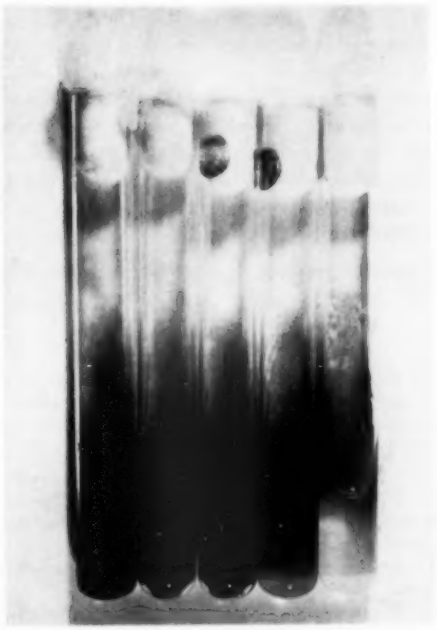


Fig. 5.—This shows persistence of pigmentation despite inhibition of growth in cultures of Pseudomonas treated with varying concentrations of mechlorethamine hydrochloride. The tube on the extreme left is the control. The concentration of mechlorethamine increases towards the right.

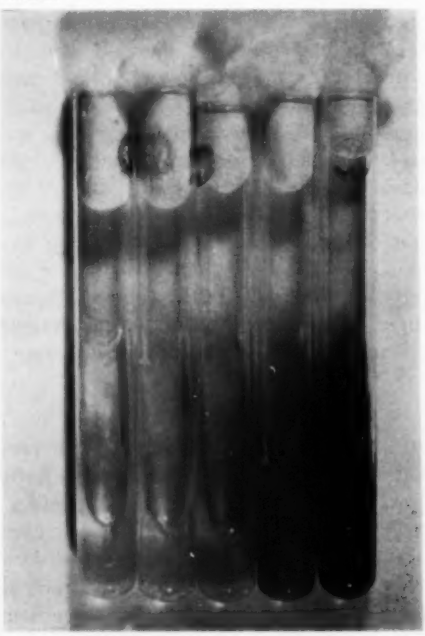
pigment can be regarded as a product of enzymatic synthetic activity, RNA can be considered basic to differentiation in bacteria as well as in other cells.

The dichotomy which exists in the effects of antitumor agents on the division and differentiation of both normal and neoplastic mammalian cells is paralleled [9] by comparable dissociations with regard to bacteria. Antibiotics, ionizing irradiation, ultraviolet irradiation, and nutritional deficiencies may affect either DNA or RNA without affecting the other or may predominantly affect one or the other. The parallel even goes as far as the production of polyploid and giant forms. The polyploid states produced by colchicine have been compared to certain effects of antibiotics, particularly the tetracyclines.[10,11]

Phenylalanine analogues [12] inhibit protein synthesis, RNA, and the formation of β-galactosidase and other inducible enzymes. Penicillin inhibits the formation of β-galactosidase, which is associated with RNA-controlled protein synthesis.[13] Conversely, Kelner [14] has shown that ultraviolet irradiation inhibits DNA but not RNA synthesis and Herriot [15] has pointed out that mechlorethamine hydrochloride inhibits DNA more than it does RNA. Webb and Nickerson [16] demonstrated that folic acid analogues caused E. coli to form filaments and decreased basophilia and DNA, although growth was not inhibited.

Loveless, Spoerl, and Weisman [17] discussed the effects of irradiation and chemical agents as regards the dichotomy between the effects of an agent on growth and on division. Folic acid antagonists were found by them to affect both growth and division, but mechlorethamine hydrochloride and triethylenemelamine, they reported, like

Fig. 6.—This shows inhibition of pigmentation despite persistence of growth in cultures of Pseudomonas treated with urethan. The tube on the extreme right is the control. The concentration of urethane increases towards the left. Pigment is reduced at the lowest concentration (second tube from right) and is absent at all the higher concentrations.



irradiation, affected primarily division. It is of interest that by their criteria colchicine and urethan were placed together, since in our work on mammalian cells there were also certain similarities in the effects of these two agents.

It is, of course, obvious that DNA and RNA synthesis are not completely independent. Chloramphenicol, for example, although it inhibits DNA but not RNA synthesis under certain conditions,[18] does inhibit the transfer to DNA of the P^{32} incorporated into the RNA until the drug is withdrawn. This is of interest in regard to our present work because we noted that pigment did develop in some of the cultures more rapidly at low concentrations of mechlorethamine hydrochloride than it did in the controls. Possibly the block described by Astrachan might result in temporarily increased synthetic activity of the processes controlled by RNA.

Cohen and Barner[19] reported that thymine deficiency resulted in a lethal imbalance between continued cytoplasmic growth of E. coli and an inhibited nuclear synthesis. They emphasized that some of the effects of antibiotics might be mediated through affecting the normally mutually interdependent balance between growth and division in micro-organisms. In an earlier paper from this laboratory[2] the suggestion was made that the cells of some tumors might retain both the capacity for division of the reserve cell and the capacity for differentiation of the postmitotic differentiating element. It was pointed out that some of the therapeutic effect of antitumor agents might therefore be the result of encouraging differentiation at the expense of division.

Summary

In Pseudomonas urethan inhibits the production of pigment disproportionately to its effect on the growth of the organisms. Mechlorethamine hydrochloride (nitrogen mustard) affects growth and pigmentation to a comparable degree, although it does interfere with growth somewhat more than it does with pigmentation. The differing effects of the drugs on the growth of Pseudomonas and on its formation of pigment are compared to the dissociated effects of antimitotic agents on the division and differentiation of mammalian cells. Certain similarities between the actions and effects of antibiotics and anticancer agents are pointed out.

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"Insulitis" in Early Juvenile Diabetes

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As noted by Warren,[1] changes in the pancreas in childhood diabetes may be expected to throw more light on the pathogenesis of the disease than those of later life. This should be particularly true of patients who die within a short time after onset.

Infiltration of the islands of Langerhans by cells, usually lymphocytes, is a rare lesion. In the absence of a diffuse pancreatitis, it is apparently specific for diabetes mellitus and is practically confined to cases of recent onset in children, although a few examples have been recorded in adolescents and young adults. It was described in the writings of some of the early students of diabetes[2-6] but received little attention until Warren[1] emphasized its occurrence in children.

Because the rarity of this lesion may be more apparent than real, and because there are interesting pathogenetic implications, it seemed worth while to describe four cases, provided by colleagues.

Report of Cases

CASE 1.—An 11-month-old boy had been unusually large at birth (by Cesarean section), weighing 11 lb. 8 oz. and measuring 23 in. The mother was subsequently found to have a normal glucose-tolerance curve, but the father's was abnormal, reaching 188 mg. % at one-half hour and 163 at one hour. The baby was healthy and responsive, with good appetite, until 11 months of age. On the morning of April 20, 1953, his mother noticed that his skin seemed "dry like parchment," and his temperature was 99.8 F. His appetite was good, but he seemed restless that night and was given ⅛ grain (8.1 mg.) of phenobarbital. The next morning he seemed fussy and was given a small dose of acetylsalicylic acid. At noon his temperature was 100.2 F, and he showed a strong desire for food, sucking on an empty bottle. The next morning, April 22, he could not hold up his head and was relatively unresponsive. He was admitted to the Elliot Community Hospital in Keene, N. H., where he was found to have an acetone odor on the breath, 4+ sugar and acetone in the urine, and blood sugar of 696 mg. %. In spite of insulin and fluids, no improvement occurred, and the child died that night.

Autopsy

The body was well developed and nourished, measuring 86 cm. and estimated to weigh 35 lb. The viscera, including the brain, were not remarkable. The pancreas weighed 8 gm. and was firm and lobulated.

Microscopically, the myocardial fibers seemed narrow and without vacuoles, although glycogen was demonstrated by the periodic acid-Schiff (PAS) method. There were small focal hemorrhages in the lungs and a fresh thrombus in a branch of a pulmonary artery. The liver cells contained glycogen and also a good deal of fat in fine vacuoles. In the kidney the distal convoluted and collecting tubules contained fine fat droplets; surprisingly, glycogen was not demonstrated. The pituitary gland appeared to be within normal limits, with numerous acidophils.

The *pancreas* was well preserved. In general, the acinar tissue showed no change, except that in some lobules it appeared more compact than elsewhere. The islands of Langerhans were not reduced in number, in fact in one section they were definitely more numerous than usual. Their appearance varied considerably from section to section and in different parts of the same section. About half of the islands showed no change other than reduction in granulation of the β-cells when stained with Gomori's aldehyde-fuchsin stain. Hydropic change was not demonstrated, either with ordinary methods or with the PAS stain. The remaining islands had a shrunken appearance, being composed of narrow cords

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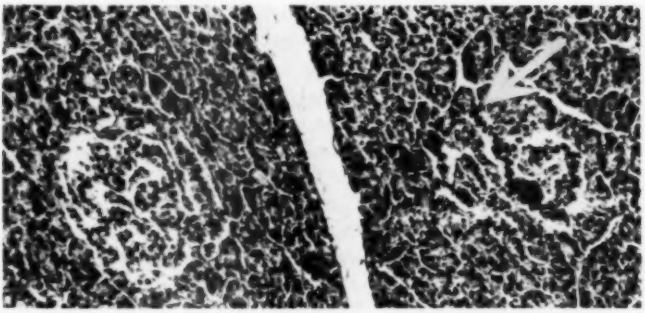

Fig. 1 (Case 1).—There are two islands of Langerhans, the one on the left essentially normal, the one on the right appearing shrunken, with some lymphocytes and apparent continuity of a cell cord with acinar tissue (arrow). The clear space in the center represents the interstitial tissue between two lobules of the pancreas; reduced about 25% from mag. × 200.

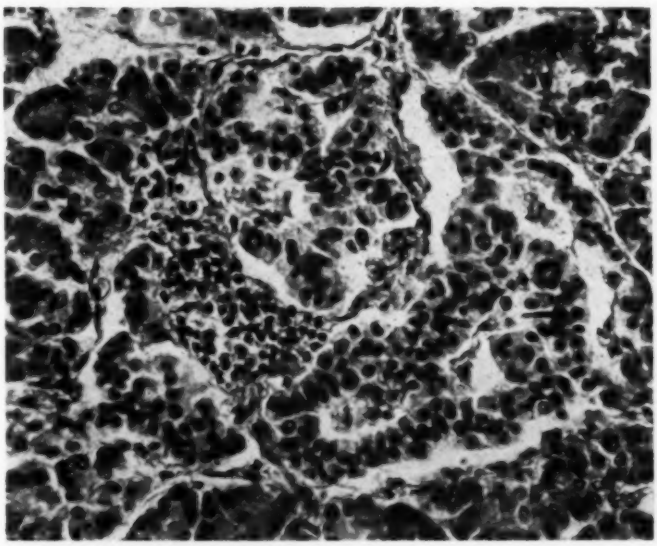

Fig. 2 (Case 1).— The upper part of this island appears fairly normal; the lower part, especially toward the left, shows, lymphocytic infiltration, with apparent shrinkage and dark staining of the cells of the island; reduced 20% from mag. × 500.

of rounded cells with hyperchromatic nuclei and scanty cytoplasm. These cells could be shown by trichrome and Bodian methods [7] to be α-cells.* In some instances both types of islands could be seen in a single microscopic field (Fig. 1). In some of the islands of the second type, an infiltration of small round cells, apparently lymphocytes, could be seen. This infiltration was sometimes in interstitial tissue within the island, sometimes in a peripheral distribution, in and around the capsule of the island. Occasionally it seemed to occur in one part of an island where the cells were of the smaller deep-staining variety (Fig. 2). There was no evidence of acute inflammation or necrosis. Occasionally, in islands of the second type, there was apparent continuity between insular and acinar cells (Fig. 1).

Case 2.—An 11-month-old white girl had been well until one week prior to admission to the North Carolina Baptist Hospital. There was no family history of diabetes. Her first symptoms were fretfulness and anorexia. Two days prior to admission she vomited and had "peculiar" respirations. A physician treated her for pneumonia without improvement, and the day before admission she continued to vomit, became lethargic, and voided large quantities of urine. She was admitted to another hospital, where the urine was found to give a 4+ reaction for both sugar and acetone. During the next 17 hours she received 1400 ml.

* Silver methods are probably not specific for α-cells,[8,9] but if a given cell shows silver-positive granules it is presumably more likely to be an α-cell than a β-cell.

of parenteral fluids and 85 units of regular insulin. The urine sugar and acetone gradually decreased to 2+ sugar and no acetone, although the blood sugar three hours before had been 596 mg. %. She did not improve and was transferred still unconscious to the North Carolina Baptist Hospital. On admission the temperature was 100.4 F; pulse, 120; respirations, 12-16; weight, 18½ lb. She was perspiring, with dilated pupils which did not react to light. The urine was negative for sugar and acetone, and the blood sugar was reported as 3 mg. %. Treatment with intravenous invert sugar and dextrose was of no avail, and she died a few hours later. Other laboratory findings before death included a leukocyte count of 23,000, with 76 segmented neutrophils and 13 nonsegmented, 8 lymphocytes, 1 monocyte, and 2 myelocytes; hemoglobin, 11.4 gm.; erythrocytes, 5,000,000; B. U. N., 24 mg. %; CO_2, 12.6 mEq. per liter; chlorides, 105.4; sodium, 129; potassium, 4.9.

Autopsy

The only significant findings in organs other than the pancreas were suppuration in the submucosa of the trachea, minimal interstitial pneumonitis (in part composed of large mononuclear cells) and minimal inflammatory changes in several other organs. However, no organisms could be cultured from the heart blood or lung.

The *pancreas*, as in the previous case, showed considerable variation in the histologic picture from section to section and from field to field. There was again an appearance of compactness in certain lobules of the gland. A minority of the islands (certainly less than half) appeared essentially negative except for loss of β-granulation. Over half of the islands were composed of narrow cords of cells, with scanty cytoplasm and condensation of nuclear chromatin similar to those in the previous case. Again these cells are assumed to be α-cells because of the absence of β-granules, although in this case silver staining was not successful. In many sections, though not in all, there was rather extensive cellular infiltration, of mixed nature, consisting largely of polymorphonuclear leukocytes, with some lymphocytes and large mononuclear cells. In this case, unlike the last, the cells involved the islands quite intimately and often extended into the interstitial tissue as well. In places a shrunken

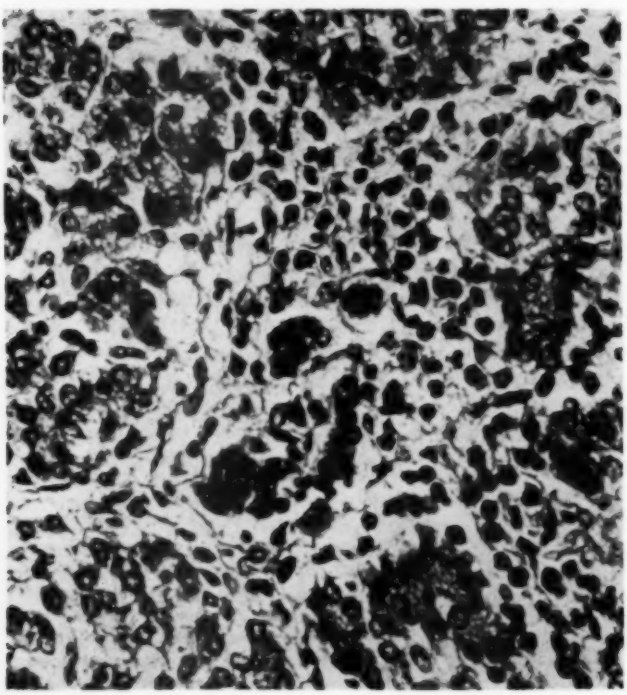

Fig. 3 (Case 2).—The remains of an island, composed of dark-staining cells with scanty cytoplasm, is surrounded by cells which are partly polymorphonuclear leukocytes, partly lymphocytes; ×600.

remnant of an island was surrounded by an abundant exudate (Fig. 3).

CASE 3.—A 9½-year-old boy had been apparently well until three nights before admission, when he wet the bed for the first time. Next day he drank a great deal of water and milk. That evening he was unusually tired and did not eat his supper. During the night he woke up three times to drink water and urinate. On the following day he began to vomit and was very thirsty. He went into coma at 4:30 a. m. on the day of admission. He died 25 minutes after admission to the Massachusetts General Hospital, the blood sugar being recorded as 772 mg. %. The patient's family history was extraordinary in that one brother and two sisters of the father had died of diabetes at the ages of 26, 16, and 17 years.

Autopsy

No significant gross findings were recorded, other than pulmonary congestion. The *pancreas* weighed approximately 20 gm. and did not appear remarkable grossly.

Microscopically, the *pancreas* showed an acute diffuse pancreatitis, with necrosis of some of the large ducts and an extensive infiltration of polymorphonuclear leukocytes in the interstitial tissue, especially involving the interlobular regions. In view of this cellular exudate, it was all the more striking to find a *lymphocytic* infiltration of many (perhaps a fourth) of the islands (Fig. 4). There was also a suggestion that this process antedated the acute pancreatitis, in that a few of the islands showed a distinct increase in connective tissue. Again, as in the two preceding cases, there was variation in the histologic picture presented by the islands. Some showed the narrow cell cords noted above, and for the most part these were the islands involved by lymphocytes. Other islands approached a "normal" appearance, but most of these showed a definite hydropic change in the β-cells (unlike the other three cases). In this case there were rare examples of apparent continuity between cells of an island and those of adjacent acini.

CASE 4.—A 17-year-old boy had been losing weight for at least two weeks and had had polydipsia and polyuria for about the same time. The latter symptoms had become severer in the last three days before admission. In spite of urging by his family, he refused to seek medical attention. On the evening before admission he began to vomit, and on the following morning he consented to go to the hospital. On admission to St.

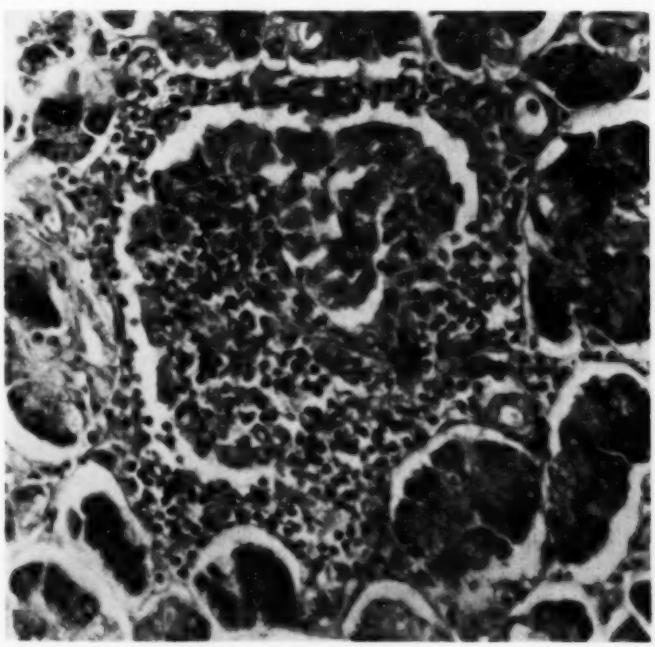

Fig. 4 (Case 3).—Extensive infiltration of lymphocytes, both within and around an island; reduced 5% from mag. × 400.

Mary's Hospital, Montreal, he was drowsy, dehydrated, and hyperventilating. The blood sugar was 468 mg. %, and plasma bicarbonate, 6.3 mEq. per liter. He was treated with fluids and insulin, but the blood pressure fell and his rectal temperature rose to 108 F. In spite of fluids, sponging, and over 1500 units of insulin in five hours, he died, still with high fever.

Autopsy

There were no significant gross findings.

Aside from an unidentified pigment in the cells of the renal tubules, the microscopic findings were confined to the pancreas. The acinar tissue did not appear remarkable, but the islands of Langerhans showed a striking and uniform type of change. They were composed of narrow, compact, ribbon-like cords of cells, similar to those described above but even more striking. The cell cords were often quite long and serpentine and occasionally seemed to merge with adjacent acinar cells. They again had a scanty, apparently homogeneous, acidophilic cytoplasm and rather deeply stained round nuclei with dense masses of chromatin. In places these cell cords were outlined and rendered more prominent by an increase of the connective tissue of the islands (Fig. 5). In addition, many (perhaps a fourth) of the islands showed lymphocytic infiltration, usually in a peripheral capsular distribution. No mitotic figures were seen, and there was no definite evidence of necrosis or hydropic change. Granule stains showed a striking predominance of α-cells, with bright-red-staining granules not only in the islet cells but also in some of the acinar cells. Most of these α-cells were also silver-positive with the Bodian stain.

Comment

As noted above, occasional cases of lymphocytic infiltration of the islands of Langerhans were observed by several of the early students of diabetes, beginning apparently with M. B. Schmidt,[2] in 1902. References are given by Kraus.[6] Sometimes hydropic change was seen, but in many cases they noted an association of the round-cell infiltration, with islands having the narrow, ribbon-like, compact structure mentioned above. In fact, the illustrations of Fischer [5] and Kraus [6] closely resemble the cases presented here. The ribbon-like structure was also described by MacCallum [10] and by Cecil.[3] These narrow cell cords, described by Weichselbaum [11] and by Kraus [6] as atrophy following hydropic degeneration, are held by Ferner [12] and Hartz [13] to be composed of α-cells, and as far as could be determined this was true in the present cases. These authors regard them as rudimentary or partial islands and not as true regeneration.

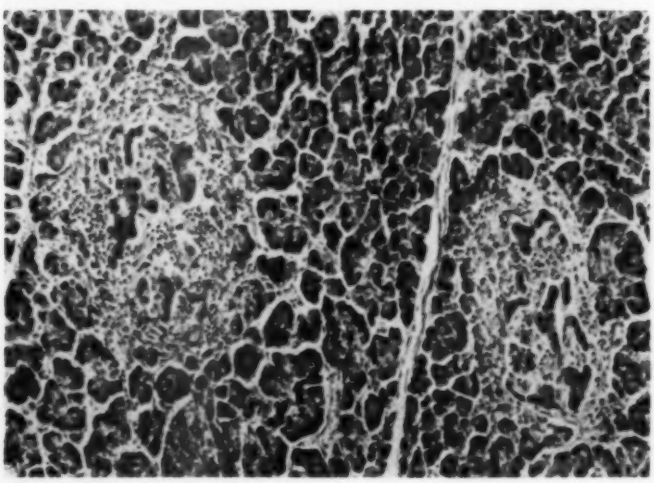

Fig. 5 (Case 4).—Two islands show lymphocytic infiltration, narrow cords of dark-staining cells, and moderate increase in connective tissue; reduced 15% from mag. × 175.

Grobéty [14] and Faller [15] described a similar phenomenon (but without lymphocytic infiltration) in rats after alloxan and regarded the cell cords as made up of α-cells which apparently multiplied by amitotic division, since they could find no mitotic figures.

In 1925, Warren and Root [16] described lymphocytic infiltration in 3 of 26 cases of diabetes. The duration of diabetes in the three cases ranged from 2 to 19 months. They remarked that the presence of some normal-appearing islands in all cases suggested an injurious agent acting over a long period of time, with regeneration of islands occurring until their power to regenerate was overcome. In 1927, Warren [1] described the pathology of 10 cases in children, most of whom had had the disease for several years, with only one showing lymphocytic infiltration. However, in the following year Stansfield and Warren [17] recorded two acute fulminating cases (one undiagnosed clinically) in children, showing a few polymorphonuclear leukocytes in the interstitial tissue and lymphocytes in the islands. One child had been drinking milk from a cow with infected udders.

The cases here presented have some of the features described by other authors, e. g., the variable appearance of the islands (Fig. 1), the narrow ribbon-like cell cords, the often peripheral distribution of the lymphocytes, and the occasional early fibrosis of an island. Hydropic change, the classical lesion of acute diabetes of recent onset, was seen in only one case but was also uncommon in the previously reported cases; this might be due to the effect of insulin in some cases, or to technical reasons.[13]

Lymphocytic infiltration of the islands is obviously a rare lesion, though perhaps not as rare as it seems (it is easily overlooked and was not mentioned in the original protocols of two of the cases here presented). Its probable importance is suggested by two facts: first, that it occurs in cases of recent onset in children and young adults, where one might hope to find evidence of the pathogenesis of the disease, and second, that it is not characteristic of the lesions found in experimental diabetes (alloxan diabetes is notable for the lack of cellular response in the islands,[18] and the various types of hormonal diabetes do not show it [19]). Also, it does not resemble the infiltration of eosinophils seen in the infants of diabetic mothers.[20,21] Therefore, one may perhaps be justified in regarding cellular infiltration of the islands as a potential clue to the nature of the injurious agent in human diabetes, at least of the "growth-onset" type.

The presence of a cellular infiltrate in the islands of Langerhans might conceivably be explained in three ways: (1) as a result of direct invasion of the islands by an infectious agent, (2) as a manifestation of functional overstimulation or "strain," (3) as the cellular reaction to damage by some unknown nonbacterial agent.

The first possibility, i. e., that of direct invasion by a bacterium or filtrable virus, gains some plausibility from the age incidence of most of the reported cases and from the very frequent association of some obvious infection, usually respiratory, with the onset of symptoms of diabetes in infants and children.[22,23] There are reported cases in which a close association between clinically typical mumps and the onset of diabetes has been noted,[24] and Gundersen [25] has claimed to find a relation between epidemics of mumps in Norway and the appearance of increased numbers of diabetics in later years. Recently the Coxsackie viruses have been shown to involve the pancreas, at least under experimental conditions.[26] Certainly cases such as those of Stansfield and Warren [17] and Case 2 of the present series, in which an intense cellular infiltration, largely of polymorphonuclear leukocytes, involved many islands, are suggestive of an active infectious process. Other cases, and this includes most of those reported, in which a mild lymphocytic infiltration affects a minority of the islands, are less convincing. An antigen-antibody reaction must also be considered. In any case,

the apparent sparing of some islands is difficult to explain.

The second hypothesis, that the cellular infiltration is a manifestation of overloading or overstimulation of the islands, was preferred by von Meyenburg,[27] who gave a fairly extensive discussion and apparently was coiner of the term "insulitis." This theory of "strain" may be criticized because of its vagueness and also because the experimental (and human) lesion usually accepted as indicative of functional overloading of the β-cells is hydropic degeneration (glycogen deposition), although doubts have been expressed.[28] It is true that lymphocytic infiltration and hydropic degeneration may occur together in the same pancreas in human diabetes (Case 3, above), but the hydropic change so characteristic of experimental diabetes due, e. g., to pituitary or adrenal hormones or partial pancreatectomy, or certain doses of alloxan, is not accompanied by cellular infiltration.[19] The viewpoint of Kraus,[6] that the islands composed of narrow cell cords, with or without lymphocytes, represent "atrophy following hydropic degeneration," is perhaps insufficiently supported.

The third possibility, that the cellular infiltrate represents a response to injury by some unknown agent, perhaps has some validity from the considerations just mentioned. Such an agent would presumably destroy the β-cells and produce enough tissue damage to elicit the accumulation of cells. Except for the lack of round-cell infiltration, the post alloxan picture described in the rat by Grobéty and Faller is similar. Sommers[29] has suggested that the breaking down of basement membranes in various organs leads to aggregations of lymphocytes. Actual disruption of basement membranes was not demonstrated in the cases presented here, but there was some evidence of splitting or reduplication of such membranes in the islands (Fig. 5).

As noted above, the lymphocytic infiltration is characteristically associated with the presence in the islands in which it occurs of narrow cords of cells with scanty cytoplasm and deeply stained nuclei, which seem to be chiefly α-cells when it is possible to do a successful granule stain and which often appear to be in continuity with acinar cells (Fig. 1). In the past these ribbon-like cords of cells have been variously regarded as representing regeneration or atrophy. It seems probable that they represent rudimentary islands and that the β-cells may be inhibited from regenerating. Some such assumption seems necessary, for instance, in Case 1, where, in view of the baby's birth weight, it is likely that the β-cells at birth had a capacity for hyperplasia which they later lost.

Conclusions

Cellular infiltration, usually lymphocytic, of the islands of Langerhans is a relatively rare but possibly significant lesion, encountered most often in diabetes of acute onset and short duration in children. Its apparent rarity may be due in part to the fact that such acute cases come to autopsy only under exceptional circumstances.

Such cellular infiltration is commonly associated with the presence, in the islands in which it occurs, of cords or ribbons of cells with scanty cytoplasm and darkly stained nuclei, which are apparently α-cells and which probably are a manifestation of injury rather than of effective regeneration.

This "insulitis" may in a few instances be a response to actual invasion of the islands by an infectious agent, although this has not been demonstrated. It is more likely the aftermath of an injury which may differ from those involved in the commonly recognized forms of experimental diabetes. It is probable that this injury in some way inhibits the regeneration of β-cells.

Drs. John D. MacAllister and H. M. Oliver, of Keene, N. H., gave permission to use Case 1; Drs. Walter Beck, Robert Prichard, and Robert Morehead, of Bowman Gray School of Medicine, Case 2; Dr. Benjamin Castleman, of the Massachusetts General Hospital, Case 3, and Drs. David Kahn and G. Joron of Montreal, Case 4.

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Histochemical Proof of Organic Phosphate Poisoning

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Since the introduction and now widespread use of the organic phosphate insecticides, many of which are extremely toxic to man, a method of proof of fatal poisoning by these agents has been sought. The organic phosphates have been the source of accidental poisoning; they are occasionally used as suicidal agents and, although not yet reported, may be expected to be employed as homicidal agents. These potent compounds all combine more or less irreversibly with the cholinesterases of the body. Therefore, one method of proving the cause of death would be to quantitate the cholinesterase activity of various tissues removed at autopsy. It would be expected that the cholinesterase levels of the tissues would be markedly depressed and in some instances all activity might be obliterated.

The determination of the true (RBC) and pseudo (plasma) cholinesterase levels of the blood has been suggested as a method applicable to one body component.[1] This has been investigated, and the applicability of this system of analysis and proof in the forensic pathology laboratory has been detailed.[2] Several years ago, in the *American Journal of Pathology*, Bergner and co-workers[3] suggested that the histochemical demonstration of true cholinesterase activity at the myoneural junction (motor end-plate area) of skeletal muscle might be a valid way to prove death due to organic phosphate poisoning. By in vitro incubation of human skeletal muscle in solutions containing various organic phosphates, she was able to demonstrate histochemically a depression or obliteration of cholinesterase activity in the motor end-plate area. Experiments were also carried out with animals poisoned and killed by various organic phosphates. Here again the enzyme activity was either markedly diminished or completely inhibited by the toxic material. No studies were available in cases of fatal organic phosphate poisoning in man. To the time of this writing no such case study has been reported.

The following case of fatal poisoning due to one of the relatively toxic organic phosphate insecticides has been studied, with use of the postmortem examination of red blood cell cholinesterase activity and the histochemical demonstration of enzyme activity at the myoneural junction in skeletal muscle.

Report of Case

A 23-year-old white man was employed by a crop-dusting company as an aircraft attendant and maintenance man. On the morning of illness and death he had been cleaning an airplane which had been used five days previously for crop dusting with Systox.* No protective measures were taken by the subject. A steam hose was used in the course of cleaning the aircraft. The man was dampened by steam and spray during the cleaning operation. In the early afternoon, he complained of headache and then became nauseated. He was given two 1/100 grain (0.65 mg.) tablets of atropine sulfate, and because of the rapid progression of symptoms he was hospitalized at 3:25 p. m. Upon the initial examination, at 3:30 p. m., he was found to be comatose and cyanotic, and to have muscular twitching. Blood pressure was 215/125 mm. Hg. The pupils were markedly constricted. There was much sweating and salivation, with mucous and exudate issuing from the nose. Moist rales were heard throughout the lung fields. An extensor type of rigidity of the muscles was noted. Several yellow stains were seen about the hands

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* Systox is a trade name for an insecticide concentrate containing 26.2% Demeton (O,O-diethyl-O-2-ethyl mercaptoethyl thiophosphate), 2.3% related organic phosphates, and petroleum distillate.

and feet. The patient was immediately treated by intratracheal suction that was productive of copious quantities of bloody fluid. Oxygen was given by mask. Atropine sulfate, 1/75 grain (0.87 mg.), was given intramuscularly, followed nearly immediately by 1/100 grain intravenously. A total of five 1/100 grain intravenous doses of the drug were given after the first intravenous injection, the last at 6:55 p. m. The patient's aeration varied from poor to good. About 5:30 p. m. multiple convulsive seizures were first noted. Intravenous sodium amobarbital in a 1 grain (65 mg.) dose was given on three occasions, but the patient's respiration gradually became more shallow, and he died at 7:20 p. m. Laboratory data were as follows: red blood cell count, 5,600,000 per cubic millimeter; hemoglobin, 17.0 gm. per 100 ml. of blood; white blood cell count, 22,500 per cubic millimeter, with 67% segmented forms, 13% lymphocytes, 18% juvenile polymorphonuclear leukocytes, and 2% myelocytes. A blood cholinesterase determination was made during the acute phase of the illness (exact time unknown). In this determination, the red blood cells had an activity of 0.18Δ pH units per 0.2 ml. per hour, and the plasma had an activity of 0.12Δ pH units per 0.2 ml. per hour. The normal range for the laboratory was given as 0.55-1.25 and 0.41-1.65Δ pH units per 0.2 ml. per hour for red blood cells and plasma, respectively (Michel's method).

An autopsy was performed at 8:45 a. m. the following day, approximately 13 hours after death. The abnormal findings are as follows: Rigor mortis was extreme. Bloody fluid was exuding from the mouth and nares. Yellow staining of both hands and the left foot was noted. The right lung weighed 560 gm., the left, 780 gm. Both lungs were congested and hemorrhagic in appearance. There was congestion of the bronchial mucosa. The airway was patent. Examination of the bowel revealed a paucity of fecal contents. The brain weighed 1600 gm., and the superficial vessels appeared congested. Microscopic examination revealed extreme dilation of all blood vessels and edema of the myocardium and lungs. No other abnormalities were noted.

At the time of autopsy, intercostal muscle was removed and placed in a dry clean bottle. Blood was withdrawn from the heart and both the blood and muscle sent to my laboratory, arriving the day after the autopsy was performed. The blood was analyzed for cholinesterase activity upon arrival, and on the following day histochemical studies of the muscle were initiated.

Methods

The method of Fleisher and Pope[4] as modified by me for determining red blood cell cholinesterase[2] was used to determine the activity of the cholinesterase of the blood sample. This particular method of analysis was chosen because of my previous experience with it in analysis of blood samples from postmortem subjects. In my hands the method yields satisfactory results with blood samples shipped without refrigeration.

To stain the true cholinesterase at the myoneural junctions in intercostal muscle, the basic method of Koelle and Friedenwald[6] modified by Gomori,[9] Bergner,[7] and by Petty and Moore[8] was employed. With this particular histochemical procedure the active cholinesterase is indicated by a brown precipitate in the region of the motor end-plate. The intercostal muscle from the presumed poisoned person and a control of similar muscle from a recently autopsied subject who had died as a result of trauma were carried through the histochemical procedure together. To prove that the staining material at the myoneural junction was dependent upon cholinesterase for its presence, samples of the subject's intercostal muscle and the control intercostal muscle were incubated for 10 minutes in 1×10^{-6} M solution of diisopropylflurophosphate (DFP) prior to staining. The absence of stained material at the motor end-plate region indicates that the cholinesterase has been completely inhibited, and the reactions upon which the histochemical procedure are based cannot proceed to the final colored product. To make certain that the organic phosphate Systox was capable of depressing cholinesterase activity at the motor end-plate areas in human muscle, in vitro studies were carried out. The intercostal muscle from a control subject was placed in a small shallow glass dish on gauze moistened with normal salt solution. A similar dish contained several cubic centimeters of technical grade Systox. Both containers were then placed in a glass moist chamber, which was sealed. The exposure of the muscle to the fumes of the organic phosphate was thus insured, and the muscle was not exposed directly to the diluent. A similar preparation was used to expose the muscle to xylene fumes to eliminate the possibility of the diluent alone causing a diminution of cholinesterase activity. Exposure of the muscle to the fumes was allowed for 18 hours.

Also, certain in vivo studies were made with use of the rat as the experimental animal. In these studies 0.5 cc. of technical Systox was given intraperitoneally to a full-grown albino rat. After the death of the animal, intercostal muscle was excised and stained for cholinesterase at the myoneural junctions. In the same staining procedure, muscle from a second rat, killed by a blow over the cervical spinal cord, served as a control.

Results

The red blood cell cholinesterase activity was 0.38μM. of acetylcholine utilized under the condition of the experiment. In our

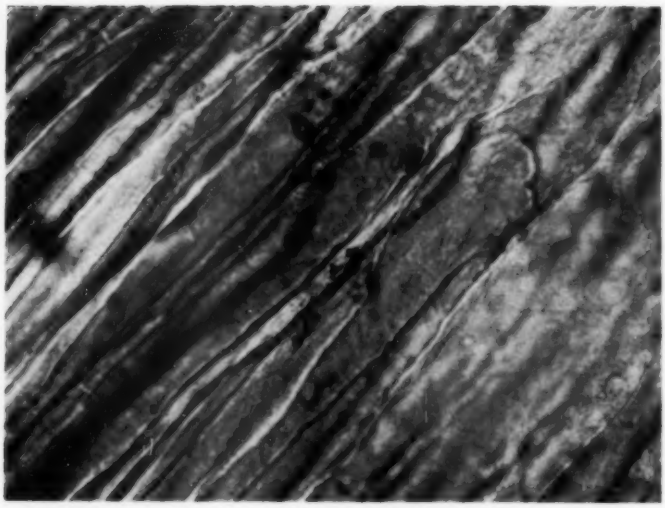

Fig. 1.—Photomicrograph of human intercostal muscle taken from person presumably fatally poisoned with Systox. Cholinesterase activity is markedly diminished, as indicated by the very light staining in the motor end-plate areas; × 165.

laboratory, the postmortem RBC cholinesterase level in 97 men who had causes of death other than organic phosphate poisoning averaged 1.52, with a range of 0.74-2.38μM. of acetylcholine utilized.[2] The level of 0.38 is lower than any observed by us, with the exception of blood from actual cases of poisoning by the organic phosphates.[9] This level is highly suggestive, if not actual proof, of such poisoning.

The results of the histochemical staining of the intercostal muscle from the subject is shown in photomicrographic form as Figure 1. The pale staining in the region of the motor endplates indicates cholinesterase activity that is much less than normal. This is particularly evident when Figure 1 is compared with Figure 2, a control preparation. No histochemical evidence of cholinesterase activity was demonstrated when muscle was exposed to DFP prior to staining.

The effects of fumes of Systox on cholinesterase activity at the myoneural junc-

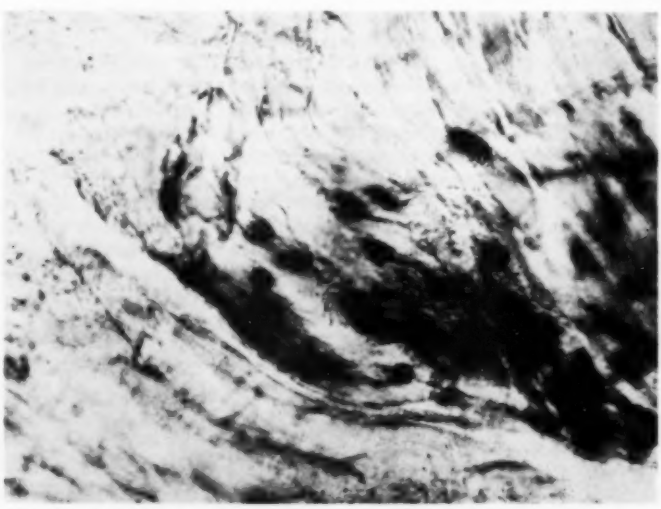

Fig. 2.—Photomicrograph of normal human intercostal muscle stained for cholinesterase activity; × 165.

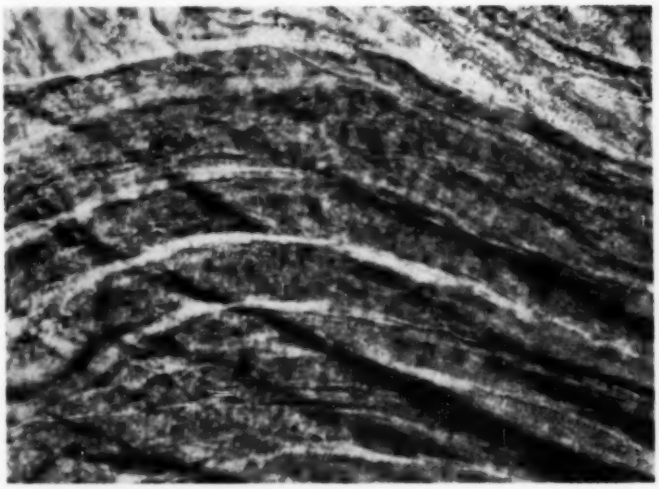

Fig. 3.—Photomicrograph of human intercostal muscle treated in vitro with Systox fumes and strained for cholinesterase activity; × 165.

tions in human muscle can be seen by examining Figure 3. It is evident that the activity of the cholinesterase is nearly obliterated, and the preparation resembles that of Figure 1.

That Systox is effective in the rat in bringing about death within 30 minutes was evident in the three instances in which this preparation was given. The animals apparently died as a result of respiratory failure following convulsive seizures, diarrhea, and muscle fasiculations. Intercostal muscle stained for motor endplate cholinesterase activity is shown in Figure 4. Muscle from a rat killed by a blow over the cervical spine is shown in Figure 5. That there is marked depletion of motor end-plate cholinesterase following death by Systox poisoning in the rat is evident by a comparison of the two figures.

Rat muscle, incubated in Systox fumes before being carried through the histochemical procedure, is shown in Figure 6. Again, depletion of the cholinesterase activity is marked.

Fig. 4.—Photomicrograph of rat intercostal muscle taken from animal killed by Systox injection. Stain for cholinesterase activity is very light; × 165.

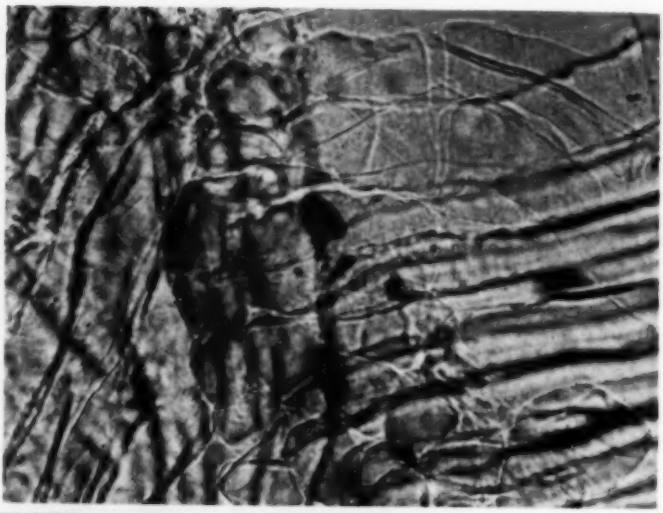

Fig. 5.—Photomicrograph of normal rat intercostal muscle stained for cholinesterase activity; × 165.

Fig. 6.—Photomicrograph of rat intercostal muscle treated in vitro with Systox fumes and stained for cholinesterase activity; × 165.

Comment

Several facts point to the cause of death due to organic phosphate poisoning. The history is compatible, and the symptomatology is typical of such poisoning.[10] The antemortem blood cholinesterase levels (both RBC and plasma) indicate poisoning by one of the organic phosphates. Such a marked depression of the RBC cholinesterase activity is never seen physiologically, and the level clearly lies outside the range of normal. No disease entity is known which will depress the cholinesterase level of the red blood cells; indeed, the red blood cell cholinesterase level is remarkably constant in any given person and not subject to great variations from the usual level.[11] One other case of human fatality due to exposure to Systox is known.[12]

If, however, the history, symptomatology and the results of the antemortem blood cholinesterase determination were not available, the postmortem blood cholinesterase level together with the histochemical studies would in themselves be adequate proof of poisoning due to exposure to one of the

potent anticholinesterase compounds. Some studies employing postmortem blood cholinesterase determinations have been reported.[1,10] To my knowledge, however, the proof of fatal human poisoning by histochemical means has never been reported in the available literature.

Proof of the cause of death due to organic phosphate poisoning is of extreme medicolegal importance. Persons who handle these materials in their daily work are exposed to an occupational hazard. Thus, their death might be compensable if it can be proved to be due to exposure to the toxic organic phosphates. A complex problem is the proof of cause of death in the instance of the crash of a crop-dusting pilot who may have lost control of his aircraft as a result of such poisoning. Many pilots have crashed to their deaths while spraying or dusting with the organic phosphates. The method outlined in this paper may give some hope to those who undertake the medicolegal investigation of such crashes.

Summary

Histochemical demonstration of diminished cholinesterase activity in the motor end-plate region in intercostal muscle from a case of fatal human poinsoning with Systox, a toxic organic phosphate preparation, was the method used to prove the cause of death. This was correlated with the postmortem red blood cell cholinesterase level. In vitro studies with human muscle and both in vitro and in vivo studies of rat muscle demonstrate that Systox is capable of depressing the cholinesterase at motor end-plates.

Lily F. Dunn, M.S., and Elizabeth J. Moore, M.A., assisted in the performance of the biochemical and histochemical procedures. Drs. Robert W. Huntington Jr., and Richard C. Dickmann provided tissues and data relevant to the case reported herein.

Louisiana State University (12).

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Whipple's Disease—Observation on Systemic Involvement

I. Cytologic Observations

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Study of the tissues of patients with Whipple's disease has shown a widespread involvement with cells containing a characteristic cytoplasmic particle. The presence of these cells in the peripheral lymph nodes allows the diagnosis to be made during life without recourse to laparotomy. The cytologic features are recorded in this paper. A survey of the pathologic findings, both gross and histologic, of five cases studied at the Henry Ford Hospital will be reported later.

Materials and Methods

These observations are based, for the most part, on paraffin sections of formalin-fixed tissue and air-dried smears and imprints. Hematoxylin and eosin stains and the Lillie short-variant periodic acid-Schiff reaction[1] were used routinely on tissue sections, and the May-Gruenwald-Giemsa stain, as well as the Schiff reaction mentioned above, were used on imprints and smears. A variety of other fixatives and stains were used in selected instances.

Observations from Histologic Sections

In 1953, while studying the nonlipid-containing histiocytes within the club-shaped villi of the small intestine and lymph nodes from an autopsy (our Case 1) I first became aware of certain previously undescribed cytologic features. In hematoxylin and eosin preparations, many of these cells, which had typical histiocytic nucleus, showed a "bubbly" or vacuolated cytoplasm (Fig. 1 *A*). The "bubbly or globular" cytoplasmic material was light blue-gray and had indistinct borders. The cytoplasm proper appeared as light eosinophilic thin strands often interwoven between the irregular masses of gray-blue material.

However, after the periodic acid-Schiff (P. A. S.) technique was applied to 6μ sections from the same paraffin block, the positive (diastase-resistant) cytoplasmic particles, while occasionally having a globular appearance, more often had a configuration suggestive of sickled erythrocytes whose ends were sharply pointed. The particles stained bright red, or magenta, and gave a more intense reaction than mucin in the intestinal glands. Furthermore, the intensity was not related to the volume of the particle. In addition to the sickle forms, there were "tear drops," hair-like threads," and other variations on a "tactoid" form. The "tactoid" particles were commonly about 2μ to 5μ in over-all length, but larger and smaller sizes were seen. For reasons described in a subsequent report, we have called these particles "sickle-form particles" and the cell containing this type of material, the sickle-form-particle-containing (S. P. C.) cell.

It should be emphasized that the sickle-form particle is *not* the only type of P. A. S.-positive nonlipid material found in these characteristic cells, e. g., amorphous globular and linear strands, of magenta-staining material can be demonstrated. Furthermore, many of these cells seem "overstuffed" with these particles, and careful examination is required to resolve the individual particles (Figs. 1*B* and *C* and 2*A* and *B*).

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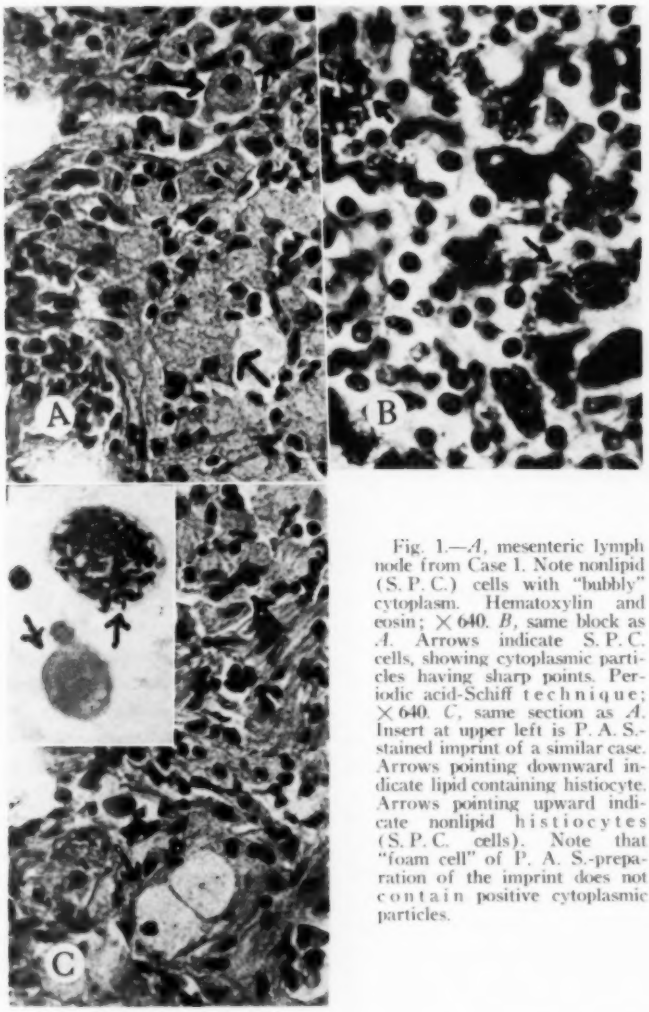

Fig. 1.—*A*, mesenteric lymph node from Case 1. Note nonlipid (S. P. C.) cells with "bubbly" cytoplasm. Hematoxylin and eosin; × 640. *B*, same block as *A*. Arrows indicate S. P. C. cells, showing cytoplasmic particles having sharp points. Periodic acid-Schiff technique; × 640. *C*, same section as *A*. Insert at upper left is P. A. S.-stained imprint of a similar case. Arrows pointing downward indicate lipid containing histiocyte. Arrows pointing upward indicate nonlipid histiocytes (S. P. C. cells). Note that "foam cell" of P. A. S.-preparation of the imprint does not contain positive cytoplasmic particles.

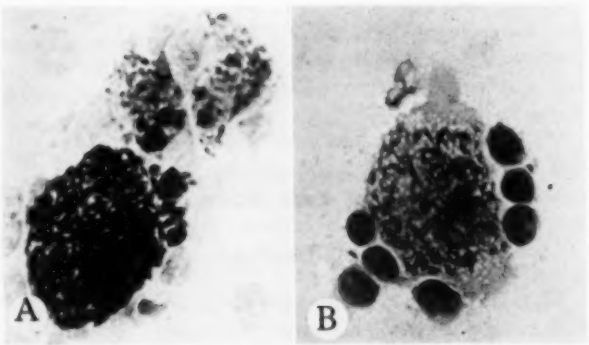

Fig. 2.—*A*, imprint of axillary lymph node from Case 3. S. P. C. cell "overstuffed" with characteristic particles. Periodic acid-Schiff technique; × 640. *B*, imprint of mesenteric node from Case 3. S. P. C. cell surrounded by lymphocytes. Note more slender sickle-form particles, as compared to *A*. This S. P. C. cell also contains uniform vacuoles, as seen in usual foam cells. Periodic acid-Schiff technique; × 640.

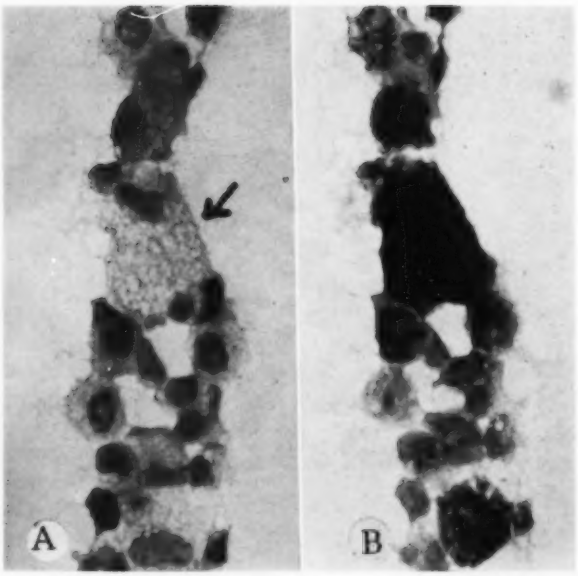

Fig. 3.—*A*, imprint of an inguinal lymph node from Case 3. Arrow indicates S. P. C. cell showing "negative images" of sickle-form particles as seen on preparation with routine Giemsa stain; × 640. *B*, same cells as in *A*, after being subjected to periodic acid-Schiff technique; × 640.

Various other tissues and organs from this case showed similar collections of S. P. C. cells, including peripheral lymph nodes and a cell block of peritoneal fluid. A study of lymph nodes and intestine from a wide variety of diseases has not yielded any findings comparable to these, which we consider specific for Whipple's disease.

In the fall of 1953, an axillary and an inguinal lymph node specimen were submitted to the department of pathology from Case 3, with a clinical diagnosis of lymphoma, ? Hodgkin's type. Routinely stained sections and imprints showed an appearance similar to that described for the autopsied case (Case 1). The periodic acid-Schiff technique confirmed these observations, and the diagnosis of Whipple's disease was rendered. A laparotomy one month later, and a subsequent necropsy, confirmed this diagnosis.

The material from this case, and others that followed, allowed us to study the S. P. C. cells in more detail, using the imprint and smear preparations of various tissues.

Observations from Imprints and Smears

In smears and imprints stained by the Romanovsky method, numerous vacuolated histiocytes were found. The nuclei of all histiocytes were similar in appearance. One group of these macrophages were the usual type of lipid-containing histiocyte "foam cell," with predominantly uniform-sized cytoplasmic vacuoles; another type, in which the apparent "vacuoles" were less distinct, varied in size and gave the cytoplasm a "moth-eaten" appearance.

When these cytologic preparations were subjected to the periodic acid-Schiff technique, these two varieties of histiocytes were again apparent. The histiocytes without lipid generally contained varying numbers of the sickle-form positive-reacting particles. Less frequently cells with uniform sized vacuoles (foam cells) showed one or more positive sickle-form particles. This was particularly prominent in those preparations containing giant cells. As in the tissue sections, not all of the visible positive particles were sickle form, and a variety of shapes, from hair-like threads to irregular globular masses (ranging up to 50μ to 60μ) were present.

Some preparations were stained with the Giemsa stain, photographed, and subsequently subjected to the P. A. S. reaction.

The same cells, which had the irregular cytoplasmic vacuoles in the Giemsa preparation, were found to contain numerous sickle-form particles (Figs. 3*A* and *B*).

Since the entire cell is examined in the imprint and smear preparations (in contrast to the tissue sections), there were more particles per cell. The irregularly shaped vacuoles which give a "moth-eaten" appearance to the S. P. C. cells were often seen to represent the nonstaining particles in material stained with hematoxylin and eosin or by the Giemsa method. This phenomenon is likewise more readily observed in imprint preparations.

Comment

Characteristic sickle-form P. A. S.-positive cytoplasmic particles have been demonstrated in mesothelial cells of the pleural, peritoneal, pericardial, and synovial lining cells. Their presence in a cell block of peritoneal fluid suggests the possibility of entertaining the diagnosis of Whipple's disease from such material. Smears stained by Papanicolau technique leave the characteristic particles unstained and show the positive P. A. S. reaction when subsequently stained appropriately. Furthermore, the positive particles are diastase-resistant.

The S. P. C. cells have been demonstrated in all of our cases of Whipple's disease. A number of these cells in various tissues and organs have led us to believe that there is a widespread involvement in this condition, as will be subsequently reported.

A study of the histologic sections from two other cases of Whipple's disease reported from different institutions * has revealed similar cells in the intestine, liver, and mesenteric, and peripheral lymph nodes.[2,3]

Summary

Cytologic studies of the material from seven cases of Whipple's disease demonstrated a characteristic periodic acid-Schiff (P. A. S.)-positive cytoplasmic particle in affected cells in all cases. The cells containing these sickle-form particles are described in some detail.

The photographs were made by Mr. Arthur Bowden.

Henry Ford Hospital.

* The material from these cases was obtained from Dr. A. J. French, Ann Arbor, Mich., and Dr. J. B. Hazard, Cleveland.

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Cerebral Mucormycosis

Report of a Case

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Cerebral mucormycosis is a rare [1-14] but distinctive entity, usually a rapidly fatal complication of diabetes mellitus. The following is a classical example, adding the 18th case to the literature.

Report of a Case

History.—This 38-year-old white married male store manager entered St. Luke's Hospital Dec. 9, 1956, for control of diabetes. His mother's father, his mother, and two maternal aunts were diabetics. The patient's sister is a diabetic taking insulin. In 1942, at the age of 24, he consulted a physician with complaints of thirst, polyuria, some weight loss, and a series of boils. His weight at that time was 135 lb. Diabetes was diagnosed, and he was placed on diet and insulin. During the next few years he did well except for moderate difficulty in maintaining a "sugar free" status without insulin reactions. Various insulins were tried, and the patient was finally stabilized satisfactorily on isophane insulin (NPH insulin). At the time of admission he was on a 2000 Cal. diet, taking 25 units of isophane insulin every 12 hours.

There was only partial peripheral vision in his left eye, resulting from a retinal tear and glaucoma due to a traumatic injury in 1936. In the spring of 1956 he was fitted with glasses for mild myopia (right eye), and in October he complained of failing vision in that eye. In the latter month arterial hypertension was discovered when he complained of passing cloudy, reddish, smoky urine, examination of which revealed red cells and a large amount of albumin. A rectal polyp showing "malignant changes" was locally excised in May, 1956.

Examination.—The patient was a well-developed, well-nourished, pallid, white man with marked generalized edema of the lower extremities. Weight was 178 lb. Head and neck were unremarkable. Eyes showed sharp disks, generalized narrowing of the arterioles, and normal veins. Many microaneurysms and many splashy hemorrhages were scattered throughout each retina with some hard yellow exudates and large splashy cotton-wool patches. The left fundus displayed a large scar of traumatic choroiditis. The opthalmologist's diagnosis was as follows: "Old retinal tear with choroiditis of left eye, mild diabetic retinopathy, and recent acute vascular hypertensive changes". The heart was slow with good tones. Lungs were entirely clear. The abdomen was negative. Pulses in the feet were good.

X-rays of the chest showed clear lungs and a normal-sized heart.

Electrocardiograph showed slight QRS slurring in Lead III and V_5, and V_5 and aV_F.

Laboratory Data.—PCV 28, RBC 3,900,000, hemoglobin 10.2 gm., WBC 7000 with 76 polymorphonuclear leukocytes (6 nonfilamented cells), 21 lymphocytes, 3 monocytes. Urine: specific gravity 1.016, albumin 3+, sugar 2+, acetone negative, 1-2 white cells, 15-20 red cells, rare hyaline casts. Serology was negative. Blood sugars fluctuated from 65 mg. % to as high as 470 mg. % during his hospital stay. Blood cholesterol 328 mg. %. Total serum proteins 4.9 gm. % (albumin 2.9, globulin 2, ratio 1.4). P. S. P. 17.5% in two hours. Addis concentration test (12 hours) showed specific gravity 1.017, albumin 3.7 gm., total volume 530 cc., WBC 6,000,000, RBC 6,460,000, and 712,500 casts, mostly granular with some RBC casts, some WBC casts, and occasional hyaline casts. Serum: creatinine 2.6 mg. %, chlorides 102 mEq., sodium 135 mEq., and potassium 5.2 mEq. Transaminase (SGOT) 10 units. Antistreptolysin titer 100 Todd units.

Course.—He was placed on a regimen of bedrest and an 1800 Cal. low-salt diabetic diet, containing 70 gm. of protein. There was rapid diuresis with a 17 lb. weight loss in five days, improvement in eyegrounds, and a drop in blood pressure from 220/130 to 170/88. Urological investigation of bladder and ureters was unremarkable with negative cultures. He was discharged on the diet with 30 units of Lente insulin once daily, with small doses of regular insulin if needed, and on vitamin B complex.

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From the Department of Pathology and Division of Medicine, St. Luke's Hospital.

The Dutton Fund of St. Luke's Hospital financed some of this patient's laboratory studies.

Discharge Diagnosis.—Diabetes mellitus, labile type, possible Kimmelstiel-Wilson kidney, and (?) subacute glomerulonephritis with arterial hypertension.

He did very well on his discharge regimen for the next several months. On returning to work, he again developed peripheral edema, easily controlled with acetazolamide (Diamox). His blood pressure remained at the level of 160/100; pulse rate was slow, and heart tones remained good. His urine continued to show albumin in large amounts, very few cellular elements, and frequent hyaline and granular casts. Serum creatinine, however, had risen to 3.5 by April 16, 1957.

Second Admission.—The patient was readmitted to St. Luke's Hospital on Sept. 4, 1957, in coma. History obtained from his private physician and family showed that one week previous he had developed sinusitis, with stuffiness in his nose, and fever. X-rays of the sinuses were reported to have shown some clouding. He was given antibiotics, but became rapidly worse. Three days later swelling of the right eye and eyelid was noted. He was given a blood transfusion. Fever continued high, and he developed difficulty in urinating, became comatose, and was transferred to St. Luke's Hospital by ambulance. At no time in his life had he had diabetic coma.

Physical Examination.—The patient was deeply comatose but not in acidosis. There were some coarse convulsive contractions of each arm. The neck was slightly stiff. There were marked proptosis of the right eye and edema of the right lid. Pupils showed a reversal of the reaction to light. Tension on the right eye was normal. There was loss of the normal cup in the right eye but no edema of the edges of the disk. Widespread narrowing of the arteries, Grade 3 and Grade 4, was present in each fundus, with engorgement of the veins, particularly on the right. The rest of the examination showed fever, diminishing of all deep reflexes, and bilateral Babinski signs. Blood pressure 205/120, pulse rate 88.

Spinal tap revealed a pressure of 570 mm. with cloudy pink fluid containing 89 white blood cells per cubic millimeter (88% polymorphonuclear leukocytes, 12% lymphocytes) and 32,250 red blood cells. Culture showed no growth. Admission blood cell count was PCV 33, RBC 3,800,000, hemoglobin 10.5 gm., WBC 37,000 with 97% polymorphonuclear leukocytes, platelets 244,000. Total serum proteins 5.5, albumin 3.1, globulin 2.4 (ratio 1.3). Urine: 2+ albumin, 5-10 red cells, 3-5 white cells, 3+ sugar, no acetone, and no casts. Serum creatinine 4.2 mg. %.

Clinical Diagnosis.—Labile diabetes, subacute glomerulonephritis, and cavernous sinus thrombosis.

Course.—The patient went rapidly downhill and became apneic. There was a rapid fall in his blood pressure, and he died Sept. 6, 1957. During his 36-hour stay in this hospital, he received antibiotic therapy, intravenous fluids, and artificial respiration terminally.

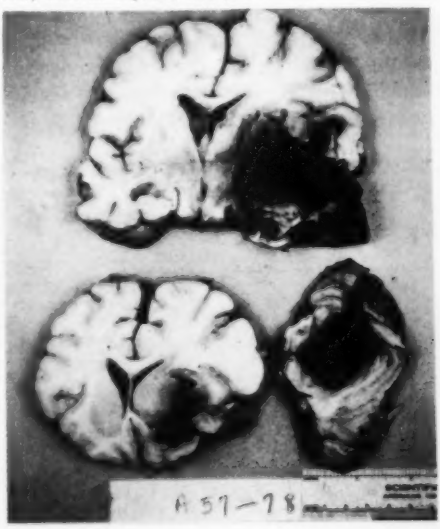
Autopsy Findings

Gross Findings

The body was well developed and well nourished. The entire upper face was edematous. Nasal and mouth passageways contained thin bloody fluid. The heart was hypertrophied (500 gm.) with a left ventricular preponderance. Coronary arteriosclerosis was moderate. The lungs exhibited patchy atelectasis and exuded minimal amounts of pink-frothy material on cut section. Major bronchi exhibited extensive reddening and granularity of the mucosa and contained much red-brown mucoid semiparticulate material. The large pale kidneys (390 gm. together) presented mild focal scarring of the surface. Pelvic and periprostatic veins contained numerous thrombi. Other viscera were grossly unremarkable.

The edematous brain (1380 gm.) demonstrated a bulging right hemisphere and a cerebellar pressure cone. Almost the entire right temporal lobe was replaced by clotted blood (Fig. 1) which protruded from the lateral surface and displaced the right basal ganglia medially. Subarachnoid hemorrhage was limited to the right temporal lobe surface, and there was no intraventricular extension.

Fig. 1.—Coronal sections of brain viewed from posterior aspect. Hemorrhage in right temporal lobe, internal capsule, and pons.



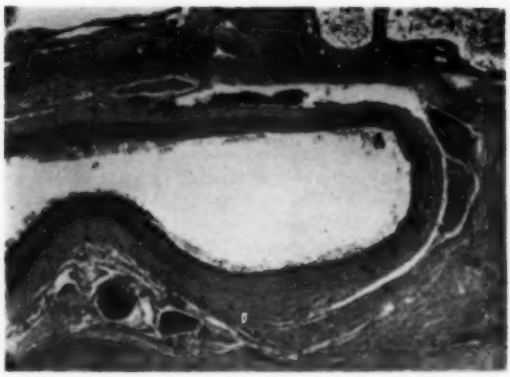


Fig. 2.—Carotid artery and cavernous sinus. Note thrombus (arrow) in the latter. The thrombus in the carotid artery has been removed. Hematoxylin and eosin; × 16.

A second hemorrhagic zone was near the midline of the cerebellar medulla on the right, extending into the upper pons. Smaller pontine hemorrhages were also apparent. Cerebral arteriosclerosis was minimal, but both internal carotid arteries contained loosely adherent thrombi, as did the superior sagittal and right lateral venous sinuses. The cavernous sinuses were not distended but contained thrombus material. The sphenoid air sinus contained mucopurulent material. Light fibrinous adhesions were noted about the optic chiasm and contiguous structures.

Microscopic Findings

Both internal carotid arteries contained unorganized thrombus attached to the intima and tangled masses of broad branching nonseptate hyphae. Numerous similar hyphae, unaccompanied by inflammatory reaction, edema, or separation of cells, invaded the muscularis of the internal carotid arteries. The cavernous sinus contained small masses of thrombus (Fig. 2) and fungal hyphae, both free and invading nerves and fibrous fasciculi in the lumen (Fig. 3). Some of the thrombus material was bland, and some was infiltrated with polymorphonuclear leukocytes. The walls of the cavernous sinus (Fig 4), the adjacent meninges and cranial nerve ganglia, and the submucosa of the sphenoid air sinus (Fig. 5) were focally infiltrated by neutrophils and some mononuclears, especially in zones where hyphae were present. Some portions of this inflammatory reaction had progressed to the formation of microabscesses. Focal acute arteritis was seen. The internal carotid arteries at the circle of Willis showed a moderate neutrophilic infiltration of the inner muscularis and intima, with fewer hyphae. The middle cerebral arteries were not thrombosed. An acute meningitis was confined to the optic chiasm area. A few hyphae lay inertly in the midbrain parenchyma above the chiasm (Fig. 6), unasso-

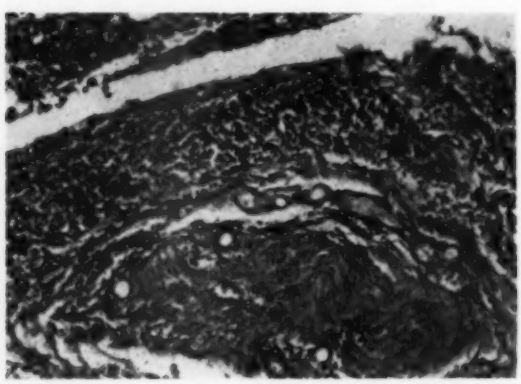


Fig. 3.—Infected thrombus about fibrous fascicle in cavernous sinus. Note hyphae. Hematoxylin and eosin; × 256.

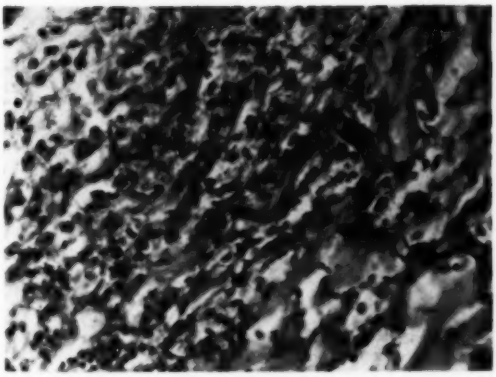


Fig. 4.—Wall of cavernous sinus showing acute inflammatory reaction and hyphae. Hematoxylin and eosin; × 400.

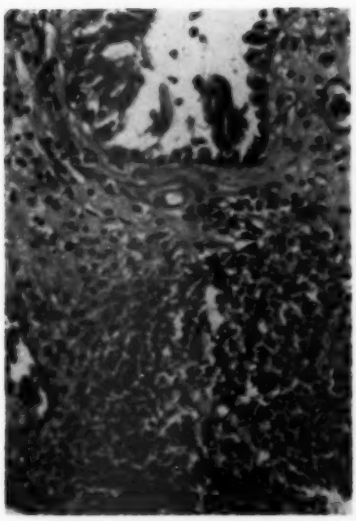


Fig. 5.—Mucous membrane of sphenoid sinus. Note hyphae in exudate in submucosa. Hematoxylin and eosin; × 256.

ciated with inflammation. None were present elsewhere in the brain. Isolated foci of acute arteritis without hyphae were noted in pontine meningeal arteries. The superior sagittal and right lateral sinus contained bland unorganized thrombi. No organisms were present in the temporal lobe hemorrhage. The latter was probably precipitated by the dural sinus thrombosis.

One bronchus was the site of an acute necrotizing ulcerating inflammation containing numerous hyphae, extending into peribronchial fat. Small arteries (Fig. 7) and veins in the inflammed area were massively invaded by hyphae, with and without thrombosis and acute vasculitis. Some hyphae invaded bronchial cartilage.

Other findings included the following: advanced diabetic glomerulosclerosis, marked renal arteriosclerosis, bilateral chronic pyelonephritis, multiple thromboses

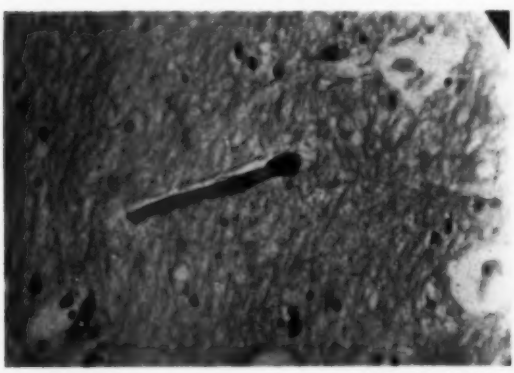


Fig. 6.—Hypha in brain. Note abscence of inflammatory reaction. Hematoxylin and eosin; × 400.

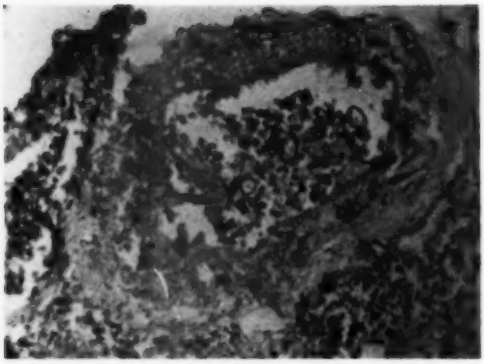


Fig. 7.—Artery in bronchial wall, showing hyphae in the lumen and in the wall. Perivascular inflammatory reaction. Hematoxylin and eosin; × 256.

of small pulmonary arteries, minimal pulmonary edema, mechanical distention of pulmonary alveoli, focal "toxic" myocarditis, acute "toxic" splenitis, and focal centrolobular liver-cell necrosis. The pancreas was unremarkable, and the rectum showed no evidence of tumor.

Cultures of material from the sphenoid sinus and the spinal fluid at autopsy grew Staphylococcus albus.

Comment

As in most of the other reported cases, the true nature of the disease was not discovered until the microscopic examination. Cultures for fungi were not made during life or at autopsy. The ethmoid sinuses and orbits were not investigated at autopsy. The evidence indicates that the infection arises in nose and paranasal sinuses and/or orbit and spreads to the brain via the internal carotid arteries and cavernous sinuses. Proptosis and ophthalmoplegia are characteristic, usually unilateral, apparently at least partly attributable to direct fungal invasion of the eye and retrobulbar space. In most cases, the mycotic infection has been limited to the head. It is likely that in this case the bronchial involvement was a separate primary focus, since there was clinical evidence of preceding orbital and sinus involvement. The hyphae have a remarkable ability to invade and penetrate tough structures, including arterial walls and even ocular sclera. Characteristically, there is total lack of inflammatory reaction to hyphae in some areas and a marked response in others. The inflammatory reaction is mainly neutrophilic and is characterized by thrombosis and vasculitis. Usually, there has been thrombosis of one or both internal carotid arteries and cavernous sinuses. This case had less extensive brain and meningeal involvement than many of the others. The meningoencephalitis involves primarily the base of the brain and inferior frontal lobes, with fibrinous meningeal adhesions and soft hemorrhagic areas in the adjacent brain. Cerebral infarcts are often present owing to the vascular occlusions caused by the fungus.

Uncontrolled diabetes has been a common denominator in most cases, but a few instances have been associated with other debilitating diseases, especially uremia and acidosis. This patient had uremia and diabetes with hyperglycemia and glycosuria but no ketosis. It is conceivable that hyperglycemia in itself may contribute to the fungal growth, since these fungi thrive on sugar. It is suggested that cortisone, antibiotics, and certain other drugs may adversely affect or precipitate this condition.[13] It is of interest that all but one of the case reports have occurred since 1943. All the cases have been rapidly fatal, with the exception of one patient treated with rigid diabetic control, iodides, and desensitization with a vaccine made from organisms cultured from the patient.[14] A diabetic patient with ethmoid sinusitis without cerebral involvement has also recovered.[13]

It has been customary to make the diagnosis of "mucormycosis" on the basis of finding the broad nonseptate branching hyphae in tissue sections. In the sense that this designation includes any members of the family Mucorales, it is correct. In at least two cases which have been cultured, the organism was found to be a member of the genus Rhizopus.[10,14] The hyphae of the genus Mucor and the genus Rhizopus appear similar in tissue, true identification resting on cultural characteristics. However, to make the diagnosis ante mortem, it is important to find the hyphae in tissues, since these fungi, being widespread in nature, are common contaminants of fungus cultures in the laboratory.[15]

Summary

A case of fatal cerebral mucormycosis is presented. Since the few reported cases have had rather stereotyped manifestations, more widespread knowledge of this condition should result in more frequent antemortem diagnoses and more thorough investigation at autopsy. It is suggested that routine examination of paranasal sinuses in diabetics coming to autopsy might help elucidate the pathogenesis.

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Estimation of Serum Calcium by Flame Photometry

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Estimation of calcium by flame photometry requires adequate photodetector sensitivity because of low intensity of light emitted at spectral lines or bands available for analysis. Interference effects due to other ions in serum (e. g., sodium, potassium, magnesium, and phosphates) have stimulated development of several flame photometric methods designed to minimize or eliminate these interference effects. The method to be described requires simple dilution of serum and standards without preliminary ashing or chemical treatment of the sample prior to analysis.

Historical Review

Severinghaus and Ferrebee[15] described a method for estimation of calcium in serum which required precipitation of serum proteins by 4% trichloroacetic acid. The supernatant protein-free fluid, a 1:10 dilution of serum, was then nebulized into an oxygen-propane-air flame. Intensity of emitted light at a wave length of 556 mμ was measured on a Beckman Model DU spectrophotometer. Aqueous standard solutions contained calcium, sodium, and potassium in a trichloroacetic acid solution in the same concentrations as in the unknown serum. Corrections for variations in sodium and potassium concentrations were made from a calibration curve set up for varying concentrations of these elements. Severinghaus and Ferrebee noted that a variation in sodium concentration (from 140 mEq. per liter) of ±14 mEq. introduced no more than 2.5% error in apparent calcium concentration; a variation in potassium concentration (from 5 mEq. per liter) of ±5 mEq. introduced no more than 0.5% error.

In the method of Mosher, Itano, Boyle, Myers, and Iseri[10] preliminary treatment of the specimen with nitric-perchloric acid and subsequent evaporation to dryness was employed to destroy plasma proteins. The residue was dissolved in hydrochloric acid, and calcium precipitated as tricalcium phosphate. The tricalcium phosphate was dissolved in hydrochloric acid and diluted to a fixed volume with a solution containing a known amount of sodium. The intensity of emitted light at 554 mμ was compared to that of a standard containing a similar concentration of sodium. A Beckman Model DU spectrophotometer with an air-oxygen-natural-gas flame was used. Since phosphates produced depression of calcium emission, sufficient diammonium hydrogen phosphate was added to standards to balance this effect.

Kapuscinski, Moss, Zak, and Boyle[7] likewise employed preliminary wet-ashing with nitric-perchloric acid to destroy protein. The residue was restored to original volume with water. Intensity of spectral emission at 554 mμ was measured on a Beckman Model DU spectrophotometer with use of an acetylene-oxygen flame, and was compared to that of an aqueous calcium standard containing sodium, potassium, magnesium, and diammonium hydrogen phosphate in concentrations approximately equal to those found in serum.

In the method of Winer and Kuhns,[16] a 1:50 dilution of serum was employed with an organic solvent containing acetone, glacial acetic acid, and Sterox (a polyoxy-

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ethylene compound) used as diluent. A synthetic standard, diluted with the same organic solvent, contained sodium, potassium, magnesium, and phosphate in concentrations similar to those found in serum. Intensity of emitted light was measured at 422.7 mμ, with use of oxyacetylene flame and a Beckman Model DU spectrophotometer with photomultiplier attachment. Results were satisfactory in their hands.

To eliminate depressant effect of phosphates on spectral emission of calcium, Denson [5] suggested passage of the solution to be analyzed through a cation-exchange column. For calcium analysis, the Beckman Model DU spectrophotometer with oxy-hydrogen flame and wave-length setting at 554 mμ was used. Values obtained for calcium and magnesium were comparable to those obtained by chemical methods. However, more calcium was recovered from the cation-exchange column than was present in the original specimen.

Brabson and Wilhide [3] removed calcium from serum by means of an ion-exchange resin. After subsequent centrifugation, washing, and resolution of the calcium-containing resin, the solution was analyzed by the internal standard method of flame photometry by comparison with aqueous standards. Results were satisfactory, in their hands, for industrial analysis.

Interfering effects of other ions were completely eliminated in the method of Rothe and Sapirstein,[13] who devised a self-standardizing method for flame photometric estimation of calcium in biological material. In this method, luminosity of a known quantity of calcium was determined under conditions of interference existing in the unknown solution by measuring increase in luminosity produced by addition of a standard amount of calcium to the unknown solution. Concentration of calcium in the unknown solution was then determined from the ratio of luminosity of an aliquot of unknown to luminosity of the known quantity of calcium. Readings were taken on a Beckman Model DU spectrophotometer with photomultiplier at 422.7 mμ, using an oxyacetylene flame.

Methods have been suggested [11,14] involving precipitation of calcium as the oxalate, with subsequent washing of the precipitate, resolution, and analysis by flame photometry. These methods offer no advantage over chemical precipitation and titration methods now in use.

Method

Standard Solution.—A 1:25 aqueous dilution of calcium standard serum is prepared. A synthetic control serum of known calcium concentration or a pooled serum, the calcium concentration of which has been determined by chemical methods, may be used.

Blank Solution.—A 1:25 aqueous dilution of a standard solution containing 140 mEq. per liter of sodium is prepared. This solution is used for the flame background zero adjustment.

Preparation of Sample.—A 1:25 aqueous dilution of the unknown serum sample is prepared.

Instrumental Conditions.—A Beckman Model B spectrophotometer with flame attachment was employed (Table 1). A Beckman Model DU spectrophotometer can similarly be used. An oxyhydrogen flame was employed, although an oxyacetylene flame can also be used. The sensitivity selector switch was placed in the 4 position. A photomultiplier phototube was used at full sensitivity (E position). Slit-width adjustment varied between 0.025 and 0.075 mm. when the 556 mμ wave-length setting was used, depending on indi-

TABLE 1.—*Instrumental Conditions*

Instrument	Beckman Model B Spectrophotometer with flame attachment & photomultiplier blue-sensitive phototube
Sensitivity setting	4 position (maximum sensitivity)
Dark current control	Varied to adjust galvanometer needle to zero while aspirating sodium blank solution
Wave length adjustment	556 mμ
Slit width adjustment	Varied to adjust galvanometer needle to setting corresponding to concentration of calcium in serum standard ×10 while calcium serum standard is aspirated
Photomultiplier selector switch	E position (maximum sensitivity)
Shutter control	Down position
Hydrogen pressure	5 ℔ (depending on performance of individual aspirator-burner assembly)
Oxygen pressure	10 ℔-15 ℔ (depending on performance of individual aspirator-burner assembly)

vidual instrumental peculiarities. Although the spectral line at 422.7 mμ and spectral band at 626 mμ are satisfactory for analysis, slit widths required at these wave lengths are approximately twice as large as that at 556 mμ and, therefore, interference effects of other ions are enhanced. All analyses were, therefore, done at 556 mμ. Oxygen and hydrogen pressures were determined by the individual characteristics of the atomizer-aspirator assembly and were adjusted to the pressures giving minimum change in per cent transmittance readings with greatest changes in gas pressure.

Procedure.—While the serum calcium standard is aspirated, the wave length is varied ("peaked") to the setting at which maximum per cent transmittance occurs. By varying the slit-width control, the galvanometer needle is then adjusted to the per cent transmittance reading corresponding to the concentration of calcium in the serum standard multiplied by 10. While the blank solution is being aspirated, the galvanometer needle is adjusted to zero per cent transmittance by varying the dark current control. These two settings are alternately adjusted until the slit-width control and dark current control settings are such that the serum calcium standard reads calcium concentration ×10 and the blank solution reads zero per cent transmittance. The 1:25 dilution of unknown serum is then introduced into the flame, and the galvanometer reading is recorded. The concentration of calcium in the unknown serum is calculated by dividing the per cent transmittance reading by 10.

Comparison with Chemical Methods

A series of 33 sera were analyzed for calcium with use of the Clark and Collip modification of the Kramer and Tisdall permanganate-titration method and the flame photometric method (Table 2). The average deviation was 0.22 mEq. per liter of calcium. In 30 of the sera, results obtained by flame photometry were higher than those obtained by the permanganate-titration method.

Denson [5] stated that values obtained for calcium were comparable to those obtained by chemical methods. Hübener [6] analyzed 20 sera by the premanganate method and by flame photometry and noted no significant difference in the two methods. On the other hand, Marquardt, Cummins, Phillips, and Fisher,[9] using the Weichselbaum-Varney Universal spectrophotometer, noted an average difference between the two methods of 0.23 mEq. per liter. This difference was not considered by them as statistically significant. Riethmüller [12] reported an average deviation of 0.23 mEq. per liter between the acidometric titration method and flame photometry, while Powell [11] found an average deviation of 0.21 mEq. per liter between the permanganate and flame photometric method. Winer and Kuhns [16] likewise noted an average deviation for calcium of 0.20 mEq. per liter when comparing their flame photometric method with the Clark and Collip method. They attributed lower values obtained by the latter method to loss of calcium during precipitation and washing procedures in the permanganate method.

TABLE 2.—*Comparison of Flame Photometric Method with Clark-Collip Method* *

Serum	Clark-Collip Method	Flame Photometry	Difference
1	5.30	5.37	0.07
2	4.62	4.84	0.22
3	4.70	4.94	0.14
4	5.06	5.00	0.06
5	4.93	5.22	0.29
6	4.30	4.45	0.15
7	4.80	5.14	0.34
8	5.00	5.35	0.35
9	6.79	7.35	0.56
10	4.90	5.28	0.38
11	5.05	5.15	0.10
12	5.10	5.30	0.20
13	5.50	5.76	0.26
14	5.00	5.15	0.15
15	5.12	5.15	0.03
16	5.02	5.15	0.13
17	4.96	5.12	0.16
18	4.96	5.15	0.19
19	5.10	5.38	0.28
20	5.02	5.40	0.38
21	4.80	4.65	0.15
22	4.90	5.05	0.15
23	4.90	5.08	0.18
24	4.96	5.28	0.32
25	4.90	5.10	0.20
26	4.80	5.10	0.30
27	5.34	5.38	0.08
28	4.30	4.58	0.28
29	4.44	4.88	0.44
30	4.64	4.75	0.11
31	4.54	4.80	0.26
32	5.10	5.08	0.02
33	4.73	4.92	0.19
		Mean deviation	0.22

*All values expressed in milliequivalents per liter of calcium.

The isotopic studies of MacIntyre[8] were considered by him to afford proof of the error arising in the precipitation method of Kramer and Tisdall and indicated that the major part of the discrepancy between the flame photometric and chemical results should be attributed to error in the chemical method. MacIntyre pointed out that normal values for serum calcium are 3%-4% higher than figures given for the precipitation method. In his series there was a mean difference of 0.28 mEq. per liter between the Kramer and Tisdall method and the flame photometric method.

Recovery Experiments

Recovery of calcium in aqueous solution added to serum calcium standards in amounts up to 5 mEq. per liter ranged from 98.2% to 101.0% with a mean recovery of 99.3% (Table 3). The per cent error falls well within the limits of error of the method and compares favorably with recovery figures given by other published flame photometric methods.[5,7,8,10,11,13,15]

Reproducibility of Results

In analyses of a replicate series of 10 sera there was a mean variation of 0.06 mEq. per liter and a standard deviation of 0.05 mEq. per liter (Table 4). This represents a maximum error of ±2% in the 95% confidence range.

TABLE 3.—*Recovery of Calcium Added to Serum Calcium Standard*

Initial Concentration of Calcium, mEq/L.	Calcium Added, mEq/L.	Total Serum Calcium, mEq/L.	Recovered	% Recovery
5.15	0.50	5.65	5.62	99.4
5.15	1.00	6.15	6.05	98.4
5.15	1.50	6.65	6.58	99.0
5.15	2.00	7.15	7.10	99.3
5.15	2.50	7.65	7.60	99.4
4.98	2.50	7.48	7.40	98.9
4.98	3.00	7.98	8.05	101.0
4.98	3.50	8.48	8.40	99.0
4.98	4.00	8.98	8.95	99.5
4.98	4.50	9.48	9.30	98.2
4.98	5.00	9.98	9.95	99.7
			Mean recovery	99.3

TABLE 4.—*Reproducibility of Results in Replicate*

Serum	Concentration of Calcium, mEq/L. I	II	Variation
1	5.37	5.33	0.04
2	4.89	4.84	0.05
3	5.00	4.94	0.06
4	5.26	5.22	0.04
5	4.45	4.50	0.05
6	5.47	5.50	0.03
7	4.90	4.90	0.00
8	5.00	5.00	0.00
9	5.36	5.20	0.16
10	4.53	4.40	0.13

Kapuscinski et al.[7] in their method, likewise achieved a standard deviation of 0.05 mEq. per liter in a series of eight determinations. Marquardt et al.[9] reported a standard deviation of 0.01 mEq. per liter with a reproducibility of 0.38%. Using the Zeiss model flame photometer, Riethmüller[12] reported a standard deviation of 0.05 mEq. per liter. The self-standardizing method of Rothe and Sapirstein[13] was reproducible to ±4%, while Severinghaus and Ferrebee[15] were able to achieve a variability of successive readings of no more than 1%.

Interference Effects

Sodium exerts a significant potentiating effect on emission of calcium.[2,6-8,10,13,15,16] Under the conditions described, this effect is most marked at 626 mμ and least marked at 556 mμ (Table 5). Increase in sodium concentration of serum of 14 mEq. per liter

TABLE 5.—*Effect of Addition of Sodium to Serum Calcium Standard on Apparent Calcium Concentrate*

Sodium Concentration, mEq/L. Initial	Added	Total	Calcium Concentration, mEq/L.	Apparent Calcium Concentration, mEq/L.	% Error
140	—	140	5.00	5.00	—
140	14	154	5.00	5.25	+ 5.0
140	28	168	5.00	5.40	+ 8.0
140	42	182	5.00	5.60	+12.0
140	56	196	5.00	5.65	+13.0
140	70	210	5.00	5.75	+15.0
140	140	280	5.00	6.40	+28.0

TABLE 6.—*Effect of Addition of Potassium to Serum Calcium Standard on Apparent Calcium Concentration*

Potassium Concentration, mEq/L.			Calcium Concentration, mEq/L.	Apparent Calcium Concentration, mEq/L.	% Error
Initial	Added	Total			
4.0	—	4.0	5.00	5.00	—
4.0	2.0	6.0	5.00	5.10	+ 2.0
4.0	4.0	8.0	5.00	5.20	+ 4.0
4.0	6.0	10.0	5.00	5.25	+ 5.0
4.0	8.0	12.0	5.00	5.35	+ 7.0

results in an apparent increase in concentration of calcium from 5 to 5.25 mEq. per liter, resulting in an error of 5.0%. Since a range of 126-154 mEq. per liter of sodium includes most variations in serum sodium concentration noted in disease states, the maximum error due to variations in sodium concentration can be assumed to be ±5%. Most errors due to smaller variations in sodium concentration will be within the limits of error of the flame photometric method.

Potassium exerts a slight but definite potentiating effect on calcium emission. Increase in serum potassium concentration from 4 to 6 mEq. per liter results in an apparent increase in calcium concentration of 0.10 mEq. per liter, corresponding to an error of 2% (Table 6). Variations in potassium concentration within the physiological range result in insignificant error.

The depressant effect of phosphates on calcium emission has been described by most observers,[1,5-7,10,13,16] although Marquardt et al.[9] stated that abnormally low and high values for phosphorus as well as magnesium, sodium, and potassium, exerted no significant effect on flame photometric estimation of calcium. Contrary to the observations of Chen and Toribara,[4] in which phosphorus exerted no significant effect in the presence of serum protein, high concentrations of phosphates produced depression of calcium emission (Table 7). An increase of from 4 to 5 mg. per 100 ml. of serum resulted in apparent decrease in serum calcium concentration from 5 to 4.65 mEq. per liter, an error of 7.0%. An increase of phosphorus concentration from 4 to 16 mg. per 100 ml. of serum resulted in apparent decrease in calcium concentration from 5.0 to 2.45 mEq. per liter, an error of 51.1%. Variations in phosphorus concentration of serum will, therefore, introduce significant error in the flame photometric method of analysis.

TABLE 7.—*Effect of Addition of Phosphorus to Serum Calcium Standard on Apparent Calcium Concentration*

Phosphorus Concentration Mg/100 Ml.			Calcium Concentration, mEq/L.	Apparent Calcium Concentration, mEq/L.	% Error
Initial	Added	Total			
4.0	—	4.0	5.00	5.00	—
4.0	1.0	5.0	5.00	4.65	− 7.0
4.0	2.0	6.0	5.00	4.10	−18.0
4.0	4.0	8.0	5.00	2.80	−44.0
4.0	6.0	10.0	5.00	2.45	−51.1
4.0	8.0	12.0	5.00	2.45	−51.1
4.0	10.0	14.0	5.00	2.45	−51.1
4.0	12.0	16.0	5.00	2.45	−51.1

Magnesium likewise exerts a depressant effect on spectral emission of calcium (Table 8). Although this effect is considerable with relatively small changes in serum magnesium concentration, the constancy of the serum magnesium level renders this possible source of error of minor importance.

Increase in serum concentration of glucose up to 300 mg. per 100 ml. resulted in no measurable change in spectral emission of calcium. Beyond this level, there was a slight apparent increase in calcium concentration due to increase in spectral emission (Table 9). The error introduced by increase in glucose concentration is within the limits of error for the flame photometric method of analysis for calcium and is apparently due to nonspecific increase in flame background due to presence of carbon.

Urea exerted no significant effect on spectral emission up to a concentration of 300 mg. per 100 ml. of serum urea nitrogen.

Comment

Flame background illumination due to presence of high concentrations of sodium

TABLE 8.—*Effect of Addition of Magnesium to Serum Calcium Standard on Apparent Calcium Concentration*

Initial Magnesium Concentration mEq/L.	Added Magnesium, mEq/L.	Total Magnesium Concentration, mEq/L.	Calcium Concentration, mEq/L.	Apparent Calcium Concentration mEq/L.	% Error
2.0	—	2.0	5.0	5.00	—
2.0	2.0	4.0	5.0	4.75	— 5.0
2.0	2.0	6.0	5.0	4.50	—10.0

is intense but can be compensated for by using, as a flame background zero adjustment, a blank solution containing sodium in a concentration approximately equal to that found in serum. Background illumination due to sodium is noted at all wave lengths of the spectrum. It is not eliminated by use of a didymium filter, which blocks out the intense spectral line of sodium at 589.3 mμ. With the galvanometer needle adjusted to zero per cent transmittance while the blank sodium solution is being aspirated, and to a value corresponding to 10 times the calcium content of the serum standard while the serum standard is being aspirated, a linear calibration curve is produced with the sodium background luminescence representing zero concentration of calcium. All calibration points for calcium fall on a line extending from the zero point through the per cent transmittance reading corresponding to the serum calcium standard multiplied by 10. Calculations are, therefore, facilitated, since calcium concentration of unknown sample can be read directly on the per cent transmittance scale.

TABLE 9.—*Effect of Addition of Glucose to Serum Calcium Standard on Apparent Calcium Concentration*

Glucose Concentration, Mg/100 Ml.			Calcium Concentration, mEq/L.	Apparent Calcium Concentration, mEq/L.	% Error
Initial	Added	Total			
100	—	100	5.00	5.00	—
100	100	200	5.00	5.00	0
100	200	300	5.00	5.00	0
100	300	400	5.00	5.10	+2.0
100	400	500	5.00	5.10	+2.0
100	500	600	5.00	5.15	+3.0
100	1,000	1,100	5.00	5.20	+4.0

A calibration curve can be constructed, but it is not necessary for routine analysis (Table 10).

Pooled serum or synthetic control serum standards are used, since aqueous synthetic standards proved to be unsatisfactory. For practical purposes, greatest economy is achieved by use of a pooled serum standard, the calcium concentration of which has been determined by chemical methods.

Adequate photodetector sensitivity is mandatory in estimation of intensity of spectral emission for calcium. At a wave length of 556 mμ, the intensity of calcium emission is about one-fiftieth that of sodium at 589.3 mμ and about one-tenth that of potassium at 767 mμ. A photomultiplier circuit for the blue-sensitive phototube is not only desirable but essential to achieve sufficient sensitivity for accurate estimation of intensity of emitted light and at the same time to allow use of as narrow slit widths as possible.

TABLE 10.—*Protocol for Construction of Calibration Curve*

Concentration of Calcium, mEq/L.	Reconstituting Fluid*	Serum Calcium Standard	Aqueous Calcium (5 mEq/L.)	Diluted to
1	0.8	0.2	—	25 ml.
2	0.6	0.4	—	
3	0.4	0.6	—	
4	0.2	0.8	—	
5	—	1.0	—	
6	—	1.0	0.2	
7	—	1.0	0.4	
8	—	1.0	0.6	
9	—	1.0	0.8	
10	—	1.0	1.0	

* Reconstituting fluid contains 140 mEq. of sodium, per liter, 4 mEq. of potassium per liter, 2 mEq. of magnesium per liter, and 4 mg. of phosphorus per 100 ml.

The smallest possible slit width consistent with adequate phototube output should be used, since background luminosity increases approximately as the square of the slit width, while spectral luminosity of the substance to be analyzed increases only proportionately. It is, therefore, apparent that interference effects due to sodium, potassium, magnesium, and phosphates are intensified with wide slit widths. High photodetector sensitivity and narrow slit widths decrease background interference to sufficiently low levels for accurate analysis. With conditions outlined in this method, the slit width at 556 mμ is about one-half that at 422.7 mμ and 626 mμ and varies from 0.025 to 0.075 mm., depending on individual instrumental peculiarities.

Flame characteristics vary considerably, depending on type of fuel used. Low flame temperature results in low excitation of orbital electrons and correspondingly low intensity of spectral emission. The oxyhydrogen flame (1800 C) is ideal for emission photometry because of spectral purity of the flame and because of low background luminosity and minimum effect of interfering ions. Although the oxyacetylene (3000 C) flame intensifies effect of interfering ions, its high temperature produces greater excitation of the calcium ion and, therefore, permits use of narrower slit widths. The narrower slit widths compensate in part for greater interference effects due to higher temperatures.

Organic solvents [16] or Sterox [13] as diluent have been used in some methods. Organic solvents increase spectral emission principally by decreasing viscosity and, therefore, increasing rate of atomization of the solution. This is, to a considerable extent, counteracted by increased cooling of the flame by the accelerated rate of atomization. Sterox is said to improve the smoothness of atomization. Neither of these two types of diluent significantly improve instrumental performance, and they were not used routinely.

A 1:25 dilution of serum is used corresponding to a final concentration of about 0.2 mEq. per liter of calcium. Calibration curves at this dilution are linear up to a concentration corresponding to 10 mEq. per liter of serum calcium or a final concentration after dilution of about 0.4 mEq. per liter. At lower dilutions linearity is not achieved. At dilutions higher than 1:50, increase in slit width necessary to produce adequate photodetector response increases flame background "noise," producing instability of the galvanometer needle and also enhancing effect of interfering ions.

Summary

A simple method for flame photometric estimation of serum calcium is described using the Beckman Model B spectrophotometer with photomultiplier phototube and with flame attachments. An oxyhydrogen flame is used.

Three wave lengths are available for analysis of calcium: a spectral line at 422.7 mμ and oxide bands at 556 mμ and 626 mμ. Under conditions described in this method, the oxide band at 556 mμ appears to give the best results.

Average deviation between the flame photometric method and the Clark and Collip permanganate-titration method is 0.22 mEq. per liter of calcium. Values obtained by flame photometry are higher than those obtained by the chemical method.

Recovery of added calcium to a standard serum calcium varies from 98.2% to 101.0%, with a mean of 99.3%.

The standard deviation of a replicate series of analyses of 10 sera is 0.05 mEq. per liter.

Sodium and potassium cause an augmentation; magnesium and phosphorus produce a depression of spectral emission of calcium. Urea up to a concentration of 300 mg. per 100 ml. of urea nitrogen produces no significant effect. Glucose up to a concentration of 300 mg. per 100 ml. has no effect. Beyond this concentration there is slight augmentation of calcium emission.

Significant error may occur in the presence of marked alteration of phosphorus levels of serum.

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Adrenal Cortical Function in Portal Cirrhosis

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The problem of adrenal cortical function in liver disease remains largely unsolved despite several studies reported in the literature. Lloyd and Williams,[20] in a study of autopsies of cases of alcoholic cirrhosis, noted decreased lipids in the adrenal cortex which was suggestive of impaired adrenal cortical function. Finestone and Shuman,[11] on the basis of the poor eosinophil response to epinephrine in patients with portal cirrhosis, have similarly concluded that there is inadequate adrenal function in this disease state.

Studies of urinary corticosteroid excretion in subjects with cirrhosis have been carried out by several workers, however, with contradictory results, mainly due to the unspecific techniques employed. Bongiovanni and Eisenmenger,[1] in a study of a group of patients with portal cirrhosis, biliary cirrhosis, and an undetermined type of cirrhosis, have found elevated urinary corticoid excretion and a normal adrenal response to corticotropin (ACTH). Peterson et al.[22] have similarly reported increased urinary "formaldehydogenic" steroids in clinically severe cases of cirrhosis. Urinary "phosphomolybdate-reducing" steroids, also designated "neutral reducing lipids," were found to be increased in subjects with cirrhosis by Shadaksharappa et al.[28] Others, however, have found urinary corticoids to be grossly normal in such cases.[15,27,30] Brown et al.,[3] in a recent study, have reported diminished urinary excretion of 17-hydroxycorticosteroids in cases of liver disease, and this, in the presence of normal levels of plasma 17-hydroxycorticosteroids, was suggestive of decreased adrenal cortical function in these subjects.

Urinary 17-ketosteroids, arising mainly from the hormones of the adrenal cortex, have been shown to be markedly diminished in clinical cases of cirrhosis.[9,11,13] Low levels of urinary androgens in these cases have also been reported.[14,35]

Studies of salt and water metabolism in subjects with cirrhosis[2,10] have shown the presence of marked abnormalities characterized by the disappearance of the normal diurnal excretion rhythm of sodium, potassium, and creatinine and low values of serum, sweat, and urinary sodium. Recent reports of excessive urinary excretion of aldosterone in cases of cirrhosis with ascites would appear largely to account for these abnormalities.[5,21]

It will be evident from this brief review of the relevant literature that the studies carried out so far on adrenal cortical function in cirrhosis have led to inconclusive results, as little light has been thrown on how far the adrenal secretion of glucocorticoids, mineralocorticoids, and androgens are individually affected in this disease. Wahi et al.,[32] in a recent study of the role of adrenal cortex in carbon-tetrachloride-induced cirrhosis in rats, have shown that adrenal function is impaired in the irreversible stage of the disease. Moreover, cortisone could cause regression of cirrhosis in the reversible stage.[33] These observations have again emphasised the need for a further investigation of the problem of adrenal cortical function in clinical liver disease. In the present study, adrenal cortical function has been assessed in human

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subjects with portal cirrhosis by a battery of adrenal function tests. Preliminary data obtained indicate that a certain proportion of cases of portal cirrhosis apparently suffer from a state of adrenal cortical insufficiency.

Materials and Methods

Forty-three cases of portal cirrhosis have been studied so far in the present investigation. The diagnosis was established on the basis of clinical findings, liver-biopsy studies, and results of liver-function tests. The liver-function tests routinely employed in these cases included (1) thymol turbidity, (2) thymol flocculation, (3) zinc sulfate turbidity, (4) colloidal gold flocculation, (5) prothrombin concentration, (6) total serum proteins and albumin, and (7) serum cholinesterase. Simultaneously, the adrenal cortical function was assessed by a series of tests as follows.

1. *Eosinophil Response to Corticotropin Administration* (Thorn Test).[31]—A minimum fall of 50% in circulating eosinophils during an interval of four hours following intramuscular injection of 25 mg. high-potency corticotropin (Organon) is taken as indicative of adequate adrenal function. The intramuscular test, though subject to criticism,[25] was adopted as it measures the immediate response of the adrenal cortex to corticotropin.

2. *Urinary Uric Acid Response to Corticotropin* (Thorn Test).[31]—A minimum rise in urinary uric acid excretion by 50% in four hours after 25 mg. of corticotropin indicates normal adrenal function.

3. *Serum Cholesterol Response to Corticotropin.*—The per cent fall in serum cholesterol in four hours following intramuscular injection of 25 mg. of corticotropin is measured. The normal response in healthy human beings was 15%-25%. The technique for cholesterol estimation was that of Zlatkis et al.[36]

4. *Fluorescein-Hyaluronidase Skin-Wheal Test.* This test was carried out as outlined by Finestone and Shuman,[11] with the modification that corticotropin was employed instead of epinephrine to measure the adrenal cortical response.

5. *Urinary 17-Hydroxycorticosteroids.*—These were determined by the technique of Reddy et al.[29,24] The normal excretion values by this technique were 5-25 mg. per 24 hours in the present study.

6. *Urinary Uropepsin.*—This has been shown to be a reliable index of adrenal cortical function in recent reports in the literature[16,17]; it was determined by the method of Gray et al.[16] Normal excretion values are 6000-25,000 units per 24 hours.

7. *Urinary 17-Ketosteroids.*—These were determined by the technique of Callow et al.[4] Normal values are 3-13 mg. per 24 hours for males and 1.5-7 mg. per 24 hours for females.

8. *Serum Sodium.*—This was estimated by the chemical method of Weinbach.[34] Normal range is 300-355 mg. per 100 ml. of serum.

9. *Urinary 17-Hydroxycorticosteroid Response to Corticotropin.*—Jenkins et al.[18] have demonstrated that the determination of urinary 17-hydroxycorticosteroids after corticotropin stimulation is a sensitive index of adrenal cortical responsiveness. De Filippis and Young[8] have adapted this into an adrenal function test in which rise in urinary 17-hydroxycorticosteroid in 24 hours after corticotropin administration is measured. In the present study rise in urinary 17-hydroxycorticosteroids in 24 hours after intramuscular administration of 25 mg. of corticotropin was measured. Normal rise: 5-10 mg. per 24 hours.

Observations

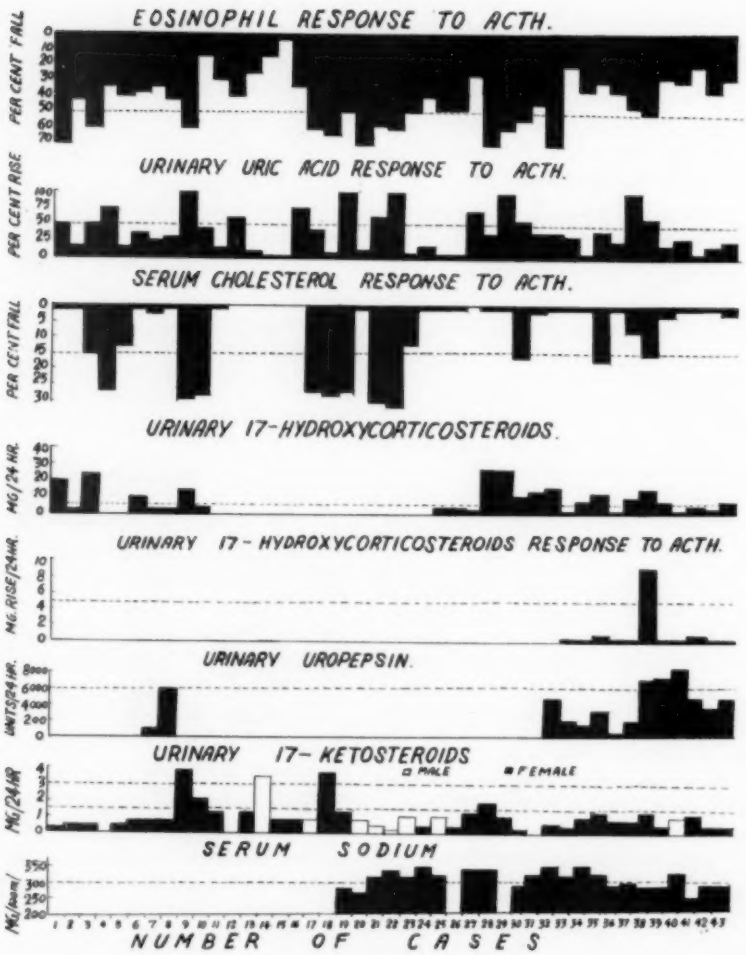
The data obtained in the adrenal-function studies in the 43 cases of portal cirrhosis studied so far are represented in the Chart. From these results it will be evident that there is diminished response to the adrenal-function tests in a proportion of the cases of cirrhosis investigated.

Eosinophil response to corticotropin is diminished in 65% of the cases studied. Lloyd and Williams[20] have similarly noted insufficient eosinophil response to corticotropin in cases of cirrhosis.

The urinary uric acid response is subnormal in 75% of the cases, and the serum cholesterol response, in 67% of the cases.

Urinary 17-hydroxycorticosteroids show diminished excretion in 44% of the cases studied; in the rest of the cases the excretion values are normal. This confirms the earlier observation of Brown et al.[3] in patients with portal cirrhosis and infectious hepatitis but contradicts the results of other studies where less specific techniques of corticoid assay were employed.[1,22,28]

Results of the urinary 17-hydroxycorticosteroid response to the corticotropin test show that there is little or no response in 91% of the cases studied. This was well correlated with diminished response to the eosinophil, urinary uric acid, and serum cholesterol response tests.



Adrenal-function study in portal cirrhosis.

Urinary uropepsin excretion is diminished in 71% of the cases investigated. Gray et al.[16] have similarly observed diminished uropepsin excretion in the cases of cirrhosis studied by them.

Urinary 17-ketosteroids are markedly decreased in the majority (88%) of the cases studied. There is no obvious correlation between 17-ketosteroid excretion and the response to the other adrenal-function tests.

Serum sodium levels are diminished in 43% of the cases; in the rest the values are within normal limits. Decreased sodium levels have been reported in cases of cirrhosis by Eisenmenger et al.[10]

The fluorescein-hyaluronidase skin-wheal test failed to give reliable results in the present study, mainly owing to difficulty in detecting end-point of the disappearance of the skin wheal, and therefore was given up. Finestone and Shuman,[12] too, have recently reported encountering similar difficulties in the use of this test.

Comment

The data presented in this paper are of a preliminary nature. However, in general,

the results obtained in the present study so far are suggestive of the presence of adrenal cortical insufficiency in a certain proportion of the cases of portal cirrhosis. In six of the adrenal-function tests employed in this study, namely, the eosinophil, urinary uric acid, serum cholesterol, urinary uropepsin, urinary 17-hydroxycorticosteroids, and urinary 17-hydroxycorticosteroid response to corticotropin tests, the responses are dependent primarily on the circulating levels of "glucocorticoid" hormones of the adrenal cortex. The insufficient response to these tests is therefore suggestive of a diminished adrenal secretion of the "glucocorticoids."

The normal levels of urinary 17-hydroxycorticosteroids found in several of the cases of cirrhosis in the present study would at first appear difficult to reconcile with diminished adrenal cortical response to corticotropin. However, impaired conversion of 17-hydroxycorticosteroids to 17-ketosteroids by the damaged liver,[6,22] as indicated by marked dimunition in urinary 17-ketosteroids, would partly account for this finding. Moreover, using essentially the same technique, DeFilippis and Young [8] have reported normal levels of urinary 17-hydroxycorticosteroids in patients with Addison's disease. This finding has led to the hypothesis that some patients with hypoadrenocorticoidism are suffering from partial rather than total inability to produce corticosteroids.

In accord with the findings in the present study is the observation that certain of the clinical features commonly seen in human subjects with cirrhosis, such as hypotension, poor tolerance to surgery, abnormal pigmentation of the skin, gastrointestinal disturbances, and disturbances in carbohydrate metabolism, are also suggestive of presence of adrenal cortical insufficiency in these patients.[29]

The exact mechanism causing this situation remains largely unknown. It is most probable, however, that it results from the state of pituitary depression which has been shown to exist in human subjects with cirrhosis.[9] Wahi et al.[32] have also provided evidence for this possibility in their studies on adrenal cortical function in carbon-tetrachloride-induced cirrhosis in rats. It is of interest in this connection that adrenal secretion of mineralocorticoids (such as aldosterone) has been demonstrated to be independent of pituitary activity [7] and to be affected by factors such as blood electrolyte levels and blood volume.[7,26] This could explain the excessive circulating levels of mineralocorticoids found in cirrhosis in presence of pituitary depression and diminished levels of glucocorticoids.

Chronic malnutrition which is invariably present in patients with cirrhosis could be another factor causing this state of adrenal cortical depression. Chronic malnutrition has been shown to devastate many of the endocrine glands, including the pituitary and adrenal cortex, so that such subjects exhibit many of the symptoms of Addison's or Simmond's disease.[19]

Summary

Adrenal cortical function has been studied in 43 cases of portal cirrhosis by means of a battery of adrenal-function tests. Preliminary data indicate that a certain proportion of these cases apparently suffer from a state of adrenal cortical insufficiency, with respect to the glucocorticoid fraction of the hormones.

Corticotropin for this study was supplied by Organon Inc.

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Constitutional Aspects of Gastric Carcinoma

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In this portion of a series of pathologic investigations of host factors in various human cancers, an attempt is made to piece together information about the hereditary, local gastric, and other tissue changes that are associated with carcinomas of the human stomach. This work was stimulated by the studies of Warren and Gates [1] and Ehrenreich [2] concerning multiple primary neoplasms and the apparent susceptibility to cancer of certain persons. To analyze the development of multiple separate cancers, investigation of the background of individual component neoplasms has appeared a prerequisite.

Material and Methods

A total of 204 autopsied cases of stomach cancer were collected, chiefly from four hospitals in the Boston area. With the permission of Drs. William A. Meissner and Shields Warren, cases were used from the New England Deaconess Hospital, and Drs. Gustave A. Dammin and Samuel P. Hicks allowed inclusion of cases from the Peter Bent Brigham Hospital. Additional cases were provided by Dr. Philip M. LeCompte, Faulkner Hospital, and Dr. Frank W. Hartman, Henry Ford Hospital. Material was also drawn from Pondville Hospital and Massachusetts Memorial Hospitals. No selection was made, and all cases with acceptable pathologic evidence of a primary gastric carcinoma were included. Clinical histories, surgical pathologic records, and slides were reviewed; all gross and microscopic data and slides from autopsies were restudied, and missing tissues were obtained when possible from stored material. Findings were recorded and tabulated for all available tissues and supplemented by cell counts of the anterior pituitary lobes and certain testicular tubular cells, using methods previously reported.[3] Pituitary counts were made without knowledge of the patient's sex or diagnosis, in conjunction with other projects. Pathologic findings were compared with control groups of over 200 men and 200 women of similar ages, who had been autopsied at the same hospitals and who had no cancer.

The age and sex distributions of the 204 cases are given in Table 1. Average age at death was 61 years for women and 65 years for men. Patients were white, with the exception of one Negro man and one Negro woman. All cases had carcinoma, either adeno or simplex.

Results

Family History.—Relatives had cancer in the cases of 13 men and 10 women, or 25% of the entire group of 60 men and 31 women with gastric cancer and adequate data. Familial cases of stomach cancer were reported three and four times, respectively (8%). Stomach cancers made up 29% of the malignant tumors reported in relatives. Cases of diabetes mellitus occurred in five and three families, respectively, or 9% of the whole cancer group, and peptic ulcers were mentioned in three families. Certain reported "cancer families" have had a frequency or preponderance of

TABLE 1.—*Distribution by Age and Sex of 204 Autopsied Gastric Cancer Cases*

Age, Yr.	Patients, No. M	F
28-40	4	6
41-50	6	9
51-60	34	10
61-70	50	19
71-80	43	15
81-92	7	0
		1
Total	144	60

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primary gastric tumors.[4-6] The relatives of patients with gastric cancer have developed from two to six times the expected number of stomach cancers, while cancers of other sites were not significantly increased.[7-9]

Blood Groups.—Of 87 cases typed in the present series, 39 were of Group O (45%), 39 of Group A (45%), 3 of Group B (3%), and 6 of Group AB (7%). Blood Group A was found in 39.7% of a control population.[10] In 52 cases Rh grouping was performed, and 43 were Rh-positive (83%).

Statistical studies have already established a significant increase of blood Group A in larger groups of gastric cancer patients.[10-13] The patients were of blood Group A in 42.08% to 53.12% of the cancer cases, in contrast to 39.7% to 45.06% for the respective control populations. Billington[14] reported that prepyloric and cardia cancer sites were significantly associated with Group A blood, and gastric fundic and body cancers were associated with Group O blood. Surgical antral and pyloric cancer cases reported by Jennings et al.[15] were Group A-related. While important theoretically, these relationships are apparent in only 3% to 20% of all gastric cancers.

Since blood groups are inherited with or without aberrant salivary or gastric secretion of blood-group-specific substances, the familial gastric cancer data were further analyzed. Of 6 such patients with blood group data, 4 were of Group A and 2 were of Group O. All six tested were Rh-positive. Familial gastric cancers are thus not regularly associated with blood Group A.*

Previous Illnesses.—Aside from those who had had childhood diseases, there were 16 patients who had inguinal hernias, including 1 woman; 14 with diabetes mellitus; 8 with hypertension; 9 with peptic ulcers, including 4 with gastric operations 4 to 24 years before death; 8 with clinical or serologic syphilis; 7 with hemorrhoids or anal fistulas; 5 with malaria; 5 with typhoid fever, and 1 woman with known pernicious anemia. The frequency of syphilis, malaria, and typhoid appeared somewhat increased; the diabetic cases were drawn from a large clinic, and pernicious anemia was not recognized clinically as frequently as anticipated.[16] In two additional women, pernicious anemia was suspected at autopsy from the presence of a megaloblastic bone marrow.

Physical Attributes.—Among 82 men with recorded body weights, 70 were normal, 12 were obese, and 10 were thin. Of 36 women with gastric cancer, 18 (50%) were obese, 14 were of normal weight, and 4 were of subnormal weight before the terminal illness. Female obesity may be related to some endocrine changes to be described later. There were 44 of 62 men and 27 of 34 women who had had children, as evidence of a normal group fertility.

Gastric Factors.—Histamine tests for gastric acidity were recorded for 50 cases, of which 34 showed no free hydrochloric acid (68% achlorhydric), 9 were hypochlorhydric, and 7 had normal gastric acidity.

The sites and Borrmann types of the specifically localized cancers are presented in Table 2. Cardia cancers and peptic ulcers were more frequent in men.

The uninvolved gastric mucosa was scrutinized, and in about 80% of 168 suitable

TABLE 2.—*Gastric Carcinoma Sites and Types*

	Men, No.*	Women, No.†
Site		
Cardia	15	3
Fundus	10	7
Body	80	33
Pylorus	29	7
Borrmann type		
I. Polypoid	25	11
II. Elevated localized	15	3
III. Ulcerated diffuse	39	8
IV Flat diffuse	37	21
Peptic ulcer	8	1
Previous gastric operations	3	1

* Total number of men is 134.

† Total number of women is 50.

* Dr. G. A. Spikes, Renger Clinic, Hallettsville, Texas, provided data for one instance of familial carcinoma in twin sisters.

cases epithelial hyperplasia was present. 1. In 40 cases (24%), accompanying the familiar intestinal metaplasia of chronic gastritis, there were irregularly crowded cystic and adenomatous foci of hyperplastic epithelium. This epithelium was either nonsecretory or mucus-producing. The altered glands were usually devoid of parietal cells. 2. In 35 cases (21%), papillary or adenomatous overgrowths of the superficial and gland-neck epithelium were observed near the openings of the gastric pits, without apparent changes in the bases of the gastric glands. 3. In 36 cases (21%), the superficial and deep types of hyperplasia were both present in the same stomach. 4. Gross pedunculated adenomatous gastric polyps with epithelial hyperplasia and intestinal metaplasia were present in 12 cases (7%) and in 4 cases were multiple. 5. Regenerative epithelial hyperplasia occurred near carcinomas arising at the margins of three typical peptic ulcers (2%) and at the stomata of three old gastroenterostomies (2%), performed for peptic ulcer therapy 13, 21, and 24 years previous. 6. Accompanying gastric cardia cancers, there was hyperplasia of the uninvolved cardia gland ducts in all five cases where ducts were identified. 7. Carcinoma in situ, mingled with hyperplasia, was found in six cases (4%), and four other gastric cancer specimens showed both epithelial hyperplasia and anaplasia.[17,18]

Miscellaneous Gastrointestinal Abnormalities.—Leiomyomas were recognized in the stomach or jejunum in three cases, and the stomach and the ileum each contained aberrant pancreas in one case. Three cases had Meckel's diverticula. Adenomatous mucosal polyps of colon were found in 16 cases (8%) and in the duodenum of 1 additional case. There were four separate primary carcinomas of the sigmoid or rectum.[19]

Hepatomegaly, with liver weights of over 1600 gm., ranging up to 2800 gm., was found in 40 men among 92 cases without liver metastases (44%) and in 6 of 30 such cases in women (20%). Among these were seven cases (6%) of hepatic parenchymal hypertrophy without other abnormalities. Hyperplasia of the intrahepatic bile duct epithelium occurred in 8 of 152 cases (5%); periportal fibrosis was common, and there were 5 instances of portal cirrhosis.

TABLE 3.—*Endocrine Alterations Accompanying Gastric Carcinoma*

	Cancerous		Noncancerous Controls	
	Incidence		Incidence	
	No. Cases	Per Cent	No. Cases	Per Cent
Hyperplasia of				
Ovarian stroma	20/31	65	98/265	37
Pituitary basophils, men	10/25	40	18/128	14
Pancreatic islets	24/177	14	24/443	5
Pancreatic ducts	21/177	12	33/443	7
Adrenal zona fasciculata	15/186	8	12/393	3
Parathyroid oxyphil adenoma, women	4/26	15	1/57	2
Thyroid hypertrophy, men	4/99	4	1/210	0.5
Hepatic hypertrophy	7/122	6	2/98	2

Endocrine Glands and Target Organs.—The major abnormalities observed are listed in Table 3. Ovarian stromal hyperplasia, while significantly frequent, was not found as regularly associated with gastric cancer as with the cancers of primary ovarian target organs, breast, and endometrium. Pituitary-cell counts indicated a significant quantitative increase of gonadotropic basophils in 10, and of acidophils in 3, of 15 male cases. The pituitary basophilism in men was not commonly accompanied by benign prostatic hypertrophy.[3] Less common abnormalities, such as hyperplasias of the pancreatic islets and ducts, thyroid hypertrophy, and parathyroid adenomas, together with the relative frequency of hepatomegaly and colonic polyps or cancers, might in part reflect responses to a common growth stimulus in some related tissues of entodermal origin.

Adrenal cortical hyperplasia and increases in pituitary hypertrophic amphophil cells, shown in Table 4, are common also in some other types of cancer and at present appear

TABLE 4.—*Average Pituitary-Cell Counts*

	Cases	Cells	Acidophils	Basophils	Amphophils	Chromophobes	HA ‡	HB §
Cancer, men	15	10,121	35.1±8.9	22.6±7.5	16.0±6.6	24.0±3.3	2.1±1.0	0.1 *
Cancer, women	5	10,504	30.0±8.9	19.9±8.9	22.1±1.9	26.5±1.2	1.3±0.3	0.2 †
Controls, men	15	10,152	38.7±7.2	18.1±2.5	20.8±5.2	21.6±1.8	0.8±0.4	<0.1
Controls, women	10	10,470	37.4±5.3	17.6±2.8	22.4±5.5	21.5±3.1	0.9±0.1	<0.1

* Three cases had 0.1%; one case, 0.4% HB cells.
† Two cases had 0.1%; one case, 0.8% HB cells.
‡ HA: Hypertrophic Amphophils
§ HB: Hyaline Basophils

to be more closely associated with the hormonal mechanisms of cancer outgrowth than with precancerous endocrine states.[3,20]

Multiple Cancers.—In the present series, 37 cases with multiple cancers had 84 primary cancers, including 2 with triple and 4 with quadruple separate neoplasms. All the triple and quadruple cases included epidermoid or basal-cell carcinomas of skin, lip, or mouth. Among 28 men, there were 11 adenocarcinomas of prostate and 4 of sigmoid or rectum; 4 epidermoid carcinomas of skin or lip, 4 of mouth or tonsil, 3 of larynx, and 2 of bladder; 2 renal-cell carcinomas; 4 basal-cell carcinomas of skin, and 1 each epidermoid carcinoma of lung, fibrosarcoma of breast, multiple myeloma and lymphosarcoma. Nine women had 3 carcinomas of breast, 3 epidermoid carcinomas of cervix, and 1 each ovarian cystadenocarcinoma, and epidermoid carcinoma of mouth and of bladder.

Among 55 similar cases previously reported by others, additional primary cancers were located as follows: in the colon, 18; breast, 10; stomach, 5; esophagus, 4; ovary, 4; skin, 4; uterus, 3; lip, tongue, and kidney, 2 each; nasopharynx, lung, prostate, and spinal cord, 1 each.[21]

The sites and types of multiple primary cancers outside the gastrointestinal tract were not unusual and did not implicate any special carcinogenic mechanism.

Comment

Gastric cancer has apparently not changed greatly in its sites, microscopic appearances, or biological behavior in the past 50 years. Hunziker's study[22] in Zürich suggested that heredity, constitution, and unknown tissue factors were of more importance than exogenous carcinogens, a search for which, incidentally, has proved rather disappointing.[23] Racial tendencies to develop stomach cancer are relatively high in Japanese, Chinese, and Scandinavian populations, intermediate in British, Canadian, and United States populations, and unusually low in the Javanese population.[24,25] Bonne and coworkers in a comparative clinicopathologic study found that the infrequency of gastric mucosal atrophy and mucinous metaplasia was morphologically the only distinctive Javanese characteristic.[26]

Relatives of gastric cancer patients investigated had nearly a twofold greater incidence of achlorhydric gastric juice, as compared to relatives of controls.[27] Comfort et al.[28] reported a subnormal mean gastric acidity and secretory activity for an average interval of 11.2 years before the development of gastric cancer, among 277 patients who averaged 48.5 years of age at the time of the first test. Eight per cent had pernicious anemia. Deaths from stomach cancer in the pernicious anemia group after 10 to 15 years were from 7.3 to 21.9 times the number expected, and in persons with achlorhydria alone the deaths from gastric cancer were 4.5 to 5.3 times the expected values.[29] Stump cancer (Stumpfcarcinom) following a reduction of gastric function produced by a gastric resection or gastroenterostomy has been thought to be 3 to 10 times more frequent than expected.[30,31]

Atrophic and metaplastic changes of "chronic atrophic gastritis" have been reported to be widespread and severe accompanying gastric cancers, but they are found

in from 30% to 82% of older noncancerous control cases.[32-36]

The causes of such degenerative changes in gastric mucosa are unexplained, but racial and familial predispositions would favor an endogenous basis. There is no proof of a sex-linked endocrine-determined tendency toward gastric cancer and, by inference, to atrophic gastritis.[22,37] Some differences in the sex incidences of gastric cancer reported may be based on institutional factors. Information concerning the endocrine aspects of gastritis is meager.

Reports which emphasize that gastric epithelial hyperplasia, atypical hyperplastic changes, and carcinoma in situ have a frequent relationship to invasive cancer are few. The conventional belief is that the stomachs of noncancerous controls of comparable ages have an equally abnormal epithelium, but this has not been supported by systematic comparative studies. Warren and Meissner[38] pointed out that atypical and undifferentiated epithelial clusters, with increased mitotic activity, developed in chronic gastritis. When severe, the hyperplasias were similar to premalignant changes in the cervix, breast, and skin. Morson[39] reported that 33% of 107 stomach cancers appeared to arise from an intestinal type of epithelium. In the present series there was hyperplasia of some uninvolved gastric mucosa in approximately 80% of the eligible cases, including 24% with intestinal metaplasia, superficial hyperplasia without metaplasia in 21%, and a mixed type of hyperplasia in 21%. Detailed controlled studies of gastric epithelial hyperplasia as a possible precursor of carcinoma are necessary, since more emphasis traditionally has been placed upon the degenerative and inflammatory aspects of "gastritis" accompanying cancer.

Factors that influence the development of gastric epithelial hyperplasias and precancerous changes are unknown. Ingested carcinogens, to which the normal gastric mucosa is impervious, might well be active when applied to an abnormally atrophic epithelium during the estimated 6- to 25-year period required for gastric cancer evolution.[29,31] The inflammatory and degenerative aspects of "chronic gastritis" might sometimes be correlated with immunologic autosensitizations to blood group substances and the subsequent development of epithelial abnormalities and a few cancers.

Gastric polyps were found in 7% of the present cancer series, comprising a less-common localized hyperplasia of precancerous significance. Apart from their acknowledged frequency in pernicious anemia, a previous study has suggested that occasionally adenomatous gastric polyps may develop secondary to pituitary stimuli, likely from acidophil cells, sometimes as part of a polyglandular endocrine hyperplastic state.[21] In a minority of the present gastric cancer cases, there were indirect indications, such as pituitary acidophil hyperplasia or hepatomegaly, that hormones might have participated in stimulating the gastrointestinal tract and other entodermal derivatives. Whether the trophic hormones arose within and acted solely upon gastrointestinal tissues and related organs or were of types already isolated is unknown.

The salient endocrine abnormalities observed appeared to be more intimately associated with the development of precancerous gastric epithelial hyperplasias than with simple gastric atrophy and metaplasia. Additional studies of host factors in persons with "gastritis" may correct this belief. The frequency of ovarian stromal hyperplasia in women, and of pituitary basophilism in men, would support some implication of gonadotropic hormones, but not in the sense of the stomach as a gonadal target organ. In this respect, there are analogies between gastric and thyroid diseases.

Once a gastric precancerous epithelial hyperplasia became established, possibly through the combined effects of an altered endocrine status and some ingested irritant, the outgrowth of most gastric cancers appeared to be associated with the same pituitary-adrenocortical hyperfunctional state

commonly encountered with other internal cancers. Knowledge of the relative importance and interrelationships of genetic, exogenous, and host factors in the development of gastric cancer is still fragmentary, and further work is needed.

Summary

In 204 autopsied cases of gastric carcinoma, the associated host factors are analyzed. Instances of stomach cancer in relatives and the proportion of cancer patients with blood Group A are greater than expected. Achlorhydria and abnormalities of the uninvolved gastric mucosa are frequent, and approximately 80% of stomach cancers are accompanied by hyperplastic epithelial changes. Irritation by ingested substances of an abnormal gastric mucosa is suspected of implication in carcinogenesis. The endocrine alterations observed favor participation of hormones in the hyperplasias and hypertrophies of certain entodermal tissues, as well as in the development of precancerous gastric epithelial hyperplasias and cancerous outgrowths.

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The Problem of Early Stromal Invasion in Carcinoma in Situ of the Uterine Cervix

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There is a "borderline" group of cases of carcinoma of the uterine cervix, lying between the diagnostic categories of invasive and in situ cancer, in which the pathologist has great difficulty in deciding whether or not invasion is present. Only rarely is he permitted by the clinician to be indecisive in his final diagnosis, however, for in large measure the therapy and prognosis offered the patient depend on the category in which the pathologist places the lesion.

During the course of a review of all cases of carcinoma in situ accessioned from 1916 through 1952 at the Free Hospital for Women (hereinafter referred to as the FHW), the "borderline" diagnosis of carcinoma in situ with gland involvement and questionable "early" stromal invasion was made in 61 of 243 cases. This report concerns histologic studies undertaken on the available material in these cases in the hope of answering the following questions:

1. What percentage of "borderline" cases with questionable "early" stromal invasion are actually invasive, and what percentage have only gland involvement?

2. Is there any difference in the prognosis for the three groups of cases: (*a*) carcinoma in situ with gland involvement, (*b*) carcinoma in situ with gland involvement and questionable "early" stromal invasion, and (*c*) carcinoma in situ with gland involvement and microscopic foci of truly invasive carcinoma?

3. Is there any point on the histological spectrum from carcinoma in situ to invasive carcinoma at which either irradiation therapy or Wertheim hysterectomy with pelvic lymphadenectomy becomes mandatory therapy?

Material and Methods

During the review mentioned above, the cases diagnosed as carcinoma in situ were divided according to the histological extent of the lesion into three groups: (1) carcinoma in situ of the endocervical or exocervical surface alone, (2) carcinoma in situ of this surface plus gland involvement, and (3) carcinoma in situ with gland involvement and questionable "early" stromal invasion. Criteria for these diagnoses have been discussed in previous reports from this hospital[1-3] and will be further amplified in future communications. Suffice it to say that in this third category were placed those cases in which microscopic examination compelled a diagnosis at least of carcinoma in situ with gland involvement but in which a definite diagnosis of invasive carcinoma could not be made.

In the series of 243 cases there were 182 cases of superficial carcinoma in situ and carcinoma in situ with glandular involvement besides the 61 cases classified as carcinoma in situ with gland involvement and questionable "early" stromal invasion. Fifteen of the sixty-one were accessioned between 1916 and 1941, during a period when celloidin sections were being prepared, and additional material was therefore not available from which to prepare sections. Of the 46 cases accessioned after 1941, paraffin blocks were found suitable for further sectioning in 25. In 21 of the 25 cases the tissue was from hysterectomy specimens, and in 4 cases the tissue was from cervical biopsy specimens. An average of 10 blocks of cervix was available from each hysterectomy specimen, with the tissue re-

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maining in each paraffin block varying from 2 to 4 mm. in thickness.

In most of the cases only one or two of the original microscopic sections from each specimen had showed foci of possible invasion. Only these "suspicious" blocks from each case were used in this study. Serial sections at 6μ were made through the selected blocks, and every fifth section was mounted and stained with hematoxylin and eosin. Periodic acid-Schiff stains were made on some of the intervening sections from each block, and the other intervening sections were discarded.

Results

Of the 25 cases in which semiserial sections were studied, microscopic foci of invasive carcinoma were found in 8 cases, including 6 hysterectomy cases and 2 biopsy cases. In 16 of the 25 cases the neoplastic epithelium was found to be confined to glandular spaces without invasion of the stroma. In one case definite stromal invasion could still not be ruled out, and the case was retained as one of carcinoma in situ with gland involvement and questionable "early" stromal invasion.

In the eight cases showing foci of invasive cancer, there were several interesting histologic features observed, including repeatedly certain patterns of stromal infiltration by the tumor cells. These same histologic patterns of focal invasion have been seen by us in the study of semiserial sections from selected cases of small frankly invasive carcinomas, and similar observations have also been reported by Stoddard [4] and by Fennell. The histologic findings in the following three cases were fairly representative of the entire group.

CASE 1.—A 30-year-old woman came to the outpatient department of the FHW on Nov. 18, 1941, complaining of pronounced dysmenorrhea. Examination disclosed the presence of white crusted areas at 10 and 2 o'clock on her cervix, and the Schiller test was positive in these areas. Biopsy was performed, and microscopic examination of the specimens was reported to show carcinoma in situ. Three weeks later, on Dec. 5, 1941, a total hysterectomy and left salpingo-oophorectomy was performed.

Twelve blocks of the cervix were made, and the original histologic diagnosis made at that time was invasive squamous-cell carcinoma of the cervix, arising in leukoplakia. After this diagnosis the patient was given several x-ray treatments followed by 2000 mg. hours of radium therapy. She was last seen in 1951, 10 years after being treated, at which time she had no evidence of recurrent disease.

Review examination in 1953 of the same slides on which the 1941 diagnosis had been made led to a slight "downgrading" of the lesion to carcinoma in situ with gland involvement and questionable "early" stromal invasion. The area of questionable invasion was in a section from the 7 o'clock block, and semiserial sections were made from this block. As illustrated in Figures 1 through 4, definite, albeit minute, foci of stromal

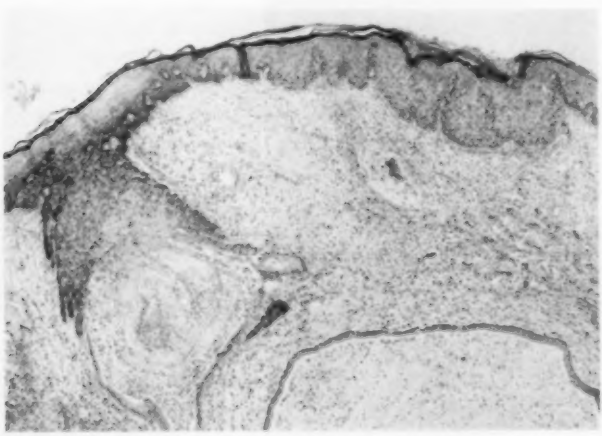

Fig. 1.—Neoplastic epithelium in gland at left in original slide prompted s e m i s e r i a l section study in Case 1. Invasion was not found there but was present beneath epithelium at right. Hematoxylin and eosin; × 35.

Fig. 2.—Epithelium at right in Figure 1, seen in section approximately 150μ deeper in block. Notice the scanty inflammatory-cell infiltrate. Hematoxylin and eosin; × 50.

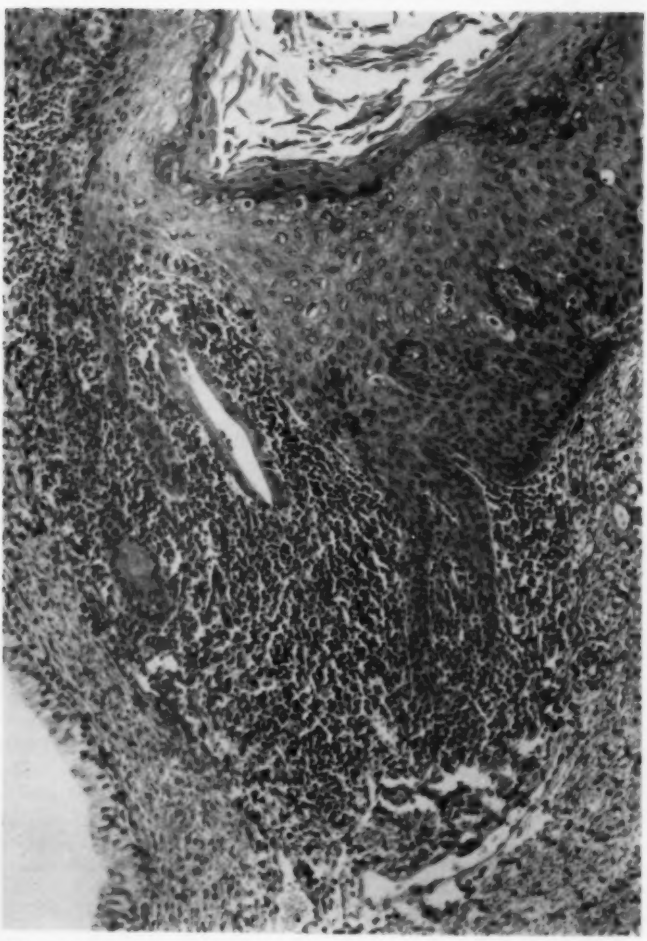

Fig. 3.—Approximately 600μ from original section in Figure 1. Notice marked chronic inflammatory-cell infiltrate around the two invading "lobster claws." Hematoxylin and eosin; reduced about 10% from mag. × 180.

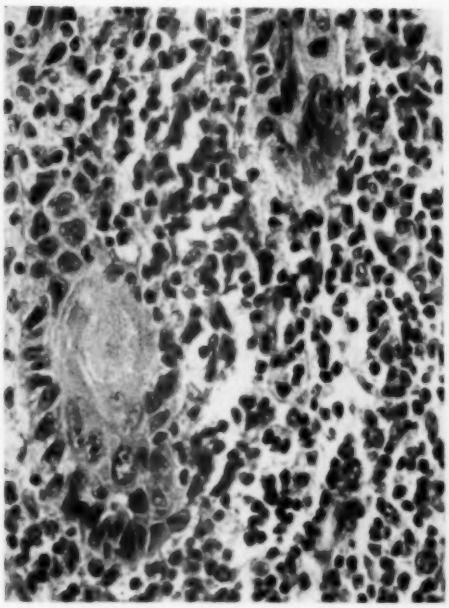

Fig. 4.—Higher magnification of well-differentiated invasive bud seen at left in Figure 3. Intercellular bridges are prominent. Above is an invasive prong of less well-differentiated cells. Most of inflammatory cells are lymphocytes. Hematoxylin and eosin; × 400.

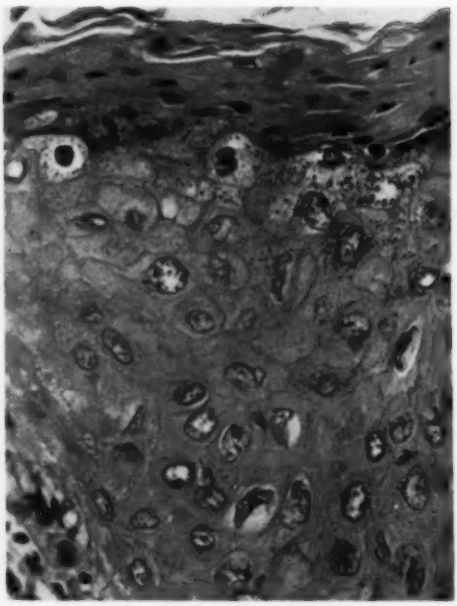

Fig. 5.—Higher magnification of central area of anaplastic surface epithelium seen in Figure 3. Notice the zones of anaplastic prickle-cell hyperplasia, "atypical Grade 1" cells, and parakeratotic cells. Hematoxylin and eosin; × 400.

invasion appeared and disappeared in less than one-half of one millimeter of tissue.

These sections illustrate a rather distinct and fairly common type of "early" stromal invasion, namely, invasion by buds of well-differentiated squamous epithelium (Fig. 4) in which intercellular bridges are readily identified. A second type of invasion is also seen in Figure 4, i. e., a prong of poorly differentiated neoplastic cells infiltrating the adjacent stroma, without basement membrane over the few cells at the tip. These tumor cells are poorly differentiated and are very different in appearance from the bud of well-differentiated cells just noted. The cells at the tip have poorly defined cell boundaries, scanty amphophilic cytoplasm, and pleomorphic nuclei. There is a heavy lymphocytic and plasma-cell infiltrate in the rather loose adjacent stroma. A comparison of Figures 2 and 3 illustrates the minimal chronic inflammatory-cell infiltrate beneath most of the nonmalignant squamous epithelium and the marked chronic inflammatory-cell infiltrate so often seen at the actual site of invasion in a field of atypical hyperplastic epithelium.

The photomicrographs from this case also illustrate several other findings of interest. First, they clearly show that invasive cancer may be present in or beneath an area of histological and clinical leukoplakia. Second, marked variation is seen both horizontally and vertically in the appearance of the hyperplastic epithelium overlying the focus of invasion. This vertical variation is well illustrated in Figure 5, wherein the superficial portion of the epithelium is anaplastic but is not definitely malignant according to usual histologic criteria. One can, in fact, see three fairly distinct types of epithelium in some areas, with neoplastic prickle cells in the lower layer, "atypical Grade 1" cells in the middle zone, and

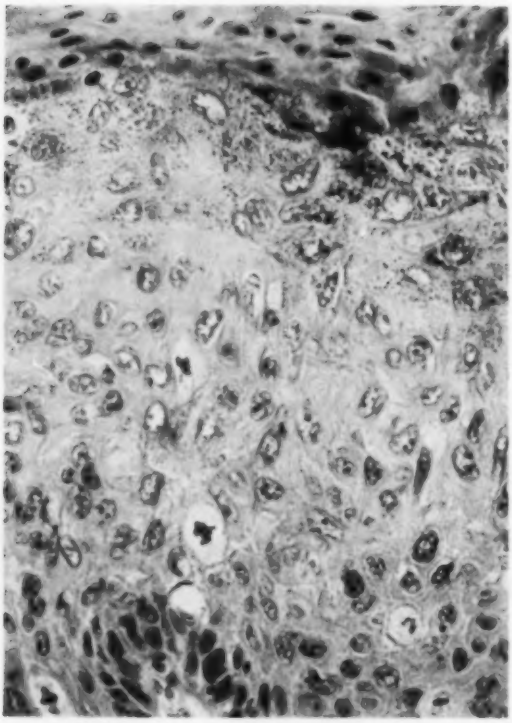

Fig. 6.—Higher magnification of neoplastic surface epithelium seen at far right in Figure 3. Cells are more pleomorphic; boundaries are less distinct, and mitoses are more numerous than in Figure 5. Hematoxylin and eosin; × 400.

somewhat anaplastic parakeratotic cells in the superficial zone. In the face of such *stratification* one cannot insist that neoplastic cells must be seen in *all* levels of squamous epithelium before a diagnosis of cancer can be made. Similarly, the *pattern* (of apparent growth) of the squamous epithelium involving the large gland shown in Figure 7 is the classic pattern of the gland involvement seen in carcinoma in situ. Although the individual cells are somewhat atypical, they do not appear to be malignant. Therefore, it seems quite possible that in these instances, as with some carcinomas of other organs, e. g., thyroid, the true malignant potential of the epithelium may not necessarily be reflected in its microscopic appearance.

Case 2.—A woman was first seen at age 34 in the FHW outpatient department, on June 29, 1948, complaining of a one-and-one-half-year history of intermenstrual spotting. She had lost 20 lb. during this period. Examination of the cervix showed only a circumoral erosion.

A vaginal smear was reported as Class 2 (atypical benign cells present), and biopsy specimens taken from 6, 9, and 11 o'clock areas were reported to show chronic cervicitis with focal areas of anaplasia. The patient returned five months later, Nov. 12, 1948. Biopsies were performed at the 1, 5, 7, and 12 o'clock areas, together with endocervical curettage. Microscopic diagnosis was chronic cervicitis. A month later (Dec. 9, 1948), the findings on examination were unchanged, and no further biopsies were performed. A vaginal smear was reported as Class 2. She returned to the clinic two months later (Feb. 3, 1949), eleven months after her initial visit, and both vaginal smear and biopsy were repeated. The former was again Class 2. The biopsy specimen, however, was reported to show carcinoma in situ with gland involvement and questionable "early" stromal invasion. When these sections were reviewed in the present study, it was agreed

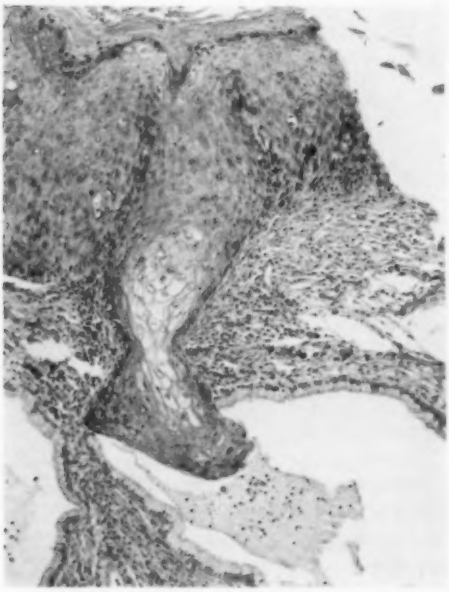

Fig. 7 (Case 1).—Extension of histologically nonmalignant epithelium into endocervical gland in typical pattern of gland involvement of carcinoma in situ. Hematoxylin and eosin; ×90.

that a diagnosis of invasive carcinoma could not be made with certainty. A month after the diagnosis of carcinoma in situ and about one year after the first clinic visit (July 6, 1949), a total hysterectomy with bilateral salpingo-oophorectomy was performed. Twelve blocks were made of the cervix. The original diagnosis on the sections made in 1949 from these blocks was carcinoma in situ with gland involvement and focal stromal invasion. The patient was last seen in 1955, six years after her hysterectomy, with no evidence of recurrence or metastasis.

On review (1953) of the original sections from the hysterectomy specimen, it was felt that there was probable, but not unequivocal, evidence of invasion in one section, while the other sections showed no invasive foci. In order either to confirm or disprove the diagnosis of early stromal invasion, semiserial sections were studied from the particular block. Beginning in the 10th of 50 sections, definite stromal invasion was found at several points, extending through approximately 1 mm. of tissue.

Of additional interest is the fact that the appearance of the invasive carcinoma here was quite different from the two types of invasion described in Case 1. The invasive buds of epithelium were quite variable in appearance, with many of the constituent epithelial cells possessing multiple nuclei or giant nuclear forms. The most striking variation from the invasive carcinoma described in Case 1, however, was seen in the stroma. More specifically, both the stroma and, to some extent, the neoplastic epithelium were heavily infiltrated with neutrophils, in addition to large numbers of plasma cells and lymphocytes. This marked acute inflammatory-cell infiltrate was seen adjacent to invasive foci both at the exoendocervical junction and those higher in the endocervix. It appeared in some way to be associated with the tumor itself rather than with any zone of actual erosion or superficial ulceration, e. g., at a biopsy site. In some areas where clusters of both plasma cells and neutrophils were seen, as in Figure 8, the former seemed to occur next to tumor cells that were fairly well demarcated from the well-vascularized, edematous stroma, whereas the neutrophils seemed to be more prominent where tumor cells were infiltrating the stroma in irregular cords and clusters. This distribution of inflammatory cells is also illustrated in Figure 10 from Case 3.

CASE 3.—A 77-year-old white woman, 22 years postmenopausal, was first seen in the FHW outpatient department on Dec. 28, 1946. For about one and one-half years she had noted protrusion of her cervix through the vaginal introitus. She had noted a pink vaginal discharge on three or four occasions in the two months prior to her first visit, and she also complained of a white mucoid vaginal discharge during this time. Urinary frequency and lower abdominal pain were other symptoms of which the patient complained. Physical examination revealed a complete procidentia with areas of mild ulceration on the cervix. Three weeks after her first visit she was admitted to the hospital and a cervicectomy, colporrhaphy, and perineorrhophy were performed.

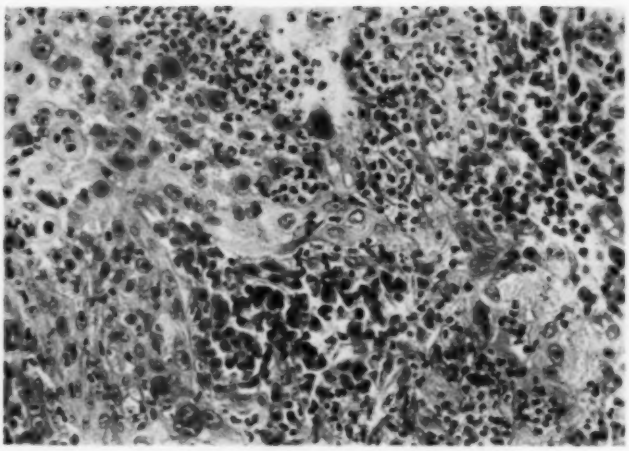

Fig. 8.—Area of invasion from Case 2. Plasma cells are present where basement membrane is intact. Neutrophils make up most of the infiltrate elsewhere. Hematoxylin and eosin; × 225.

The original pathology report on the specimen was "squamous carcinoma in situ with gland involvement and probable early stromal invasion." The line of resection was reported to be free of tumor. The patient was last seen seven years after her surgery, at which time there was no evidence of recurrence or metastasis.

On review of the sections in 1953, we concurred with the original diagnosis, and semiserial sections were therefore made. Within a few sections, foci of invasion were found along the edge of an old endocervical laceration (Fig. 9). In the same section foci of invasion were also found high in the endocervix in a field of extensive in situ carcinoma (Fig. 10).

Of interest to clinician is the fact that this represents a case of invasive carcinoma in procidentia. In addition, this patient was apparently cured by cervicectomy. Of interest to the pathologist is that, as in Case 1, this represents neoplastic epithelium in, or adjacent to, a zone of both clinical and histological leukoplakia. A further similarity between this case and Case 1 is seen in the appearance of the invasive prong shown in Figure 9, from which small clusters of tumor cells become isolated in later sections. Unlike Case 1, however, this prong does not arise directly from well-differentiated squamous epithelium or an epidermoid pearl but from only moderately well-differentiated epithelium. This finding

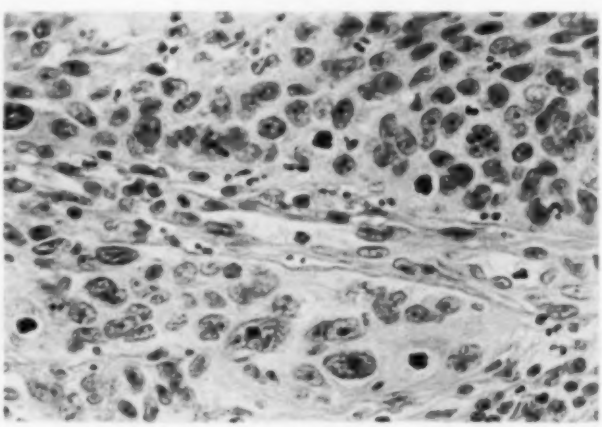

Fig. 9.—Prong of poorly differentiated neoplastic epithelium in Case 3, seen at site of invasion in healed laceration. Superficial cells in this area were well differentiated and heavily keratinized. Notice plasma cells in stroma. Hematoxylin and eosin; × 400.

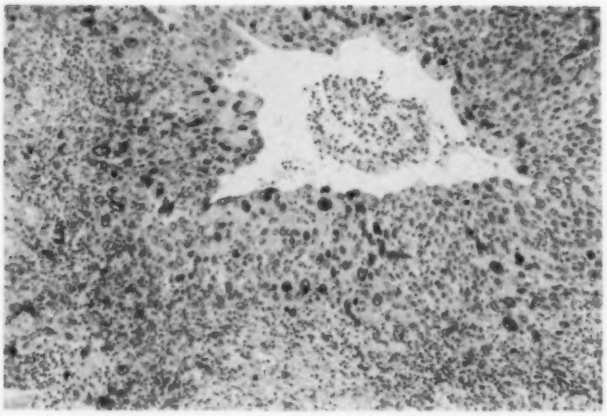

Fig. 10 (Case 2).—Area of diffuse invasion around a gland higher in endocervix. Notice large, often bizarre, nuclei of epithelial cells and heavy acute and chronic inflammatory cell infiltrate. Hematoxylin and eosin; × 100.

points up the fact that the actual histologic form, including the degree of differentiation taken by neoplastic epithelium in foci of invasion, does not necessarily resemble the malignant epithelium in the larger field of carcinoma in situ from which the foci originate.

All of the 25 cases in which semiserial sections were studied have been followed five or more years after therapy. In none of the eight cases in which invasive foci were found has there been any evidence of recurrence or of metastasis. Two patients had irradiation therapy, one had a cervicectomy, and five had hysterectomies. Similarly, after five or more years of follow-up, there has been no recurrence or metastasis reported in the 17 cases in which no foci of invasive carcinoma were found.

Likewise, there have been no deaths, recurrences, or metastases from carcinoma of the cervix in the group of 15 patients with questionable "early" stromal invasion seen prior to 1941 on whom celloidin blocks were available and none in the group seen after 1941 in which the paraffin blocks were either unavailable or unsuitable for serial sectioning.

Comment

In answer to the first question posed in the introduction, "What percentage of 'borderline' cases with questionable 'early' stromal invasion are actually invasive, and what percentage have only gland involvement?" we found that 8 (32%) out of 25 cases of carcinoma in situ with gland involvement and *questionable "early" stromal invasion* had microscopic foci of invasive carcinoma when the tissue was studied further. Sixteen cases (64%) had only gland involvement, and one case (4%) remained in the "borderline" category despite the semiserial sections. If these figures are applied to the 36 cases of questionable "early" stromal invasion in which serial sections could not be made, 12 of them might also have had invasive foci. Thus 20 of the 61 "borderline" cases may have represented invasive carcinoma of minimal extent, while in the remainder the neoplastic epithelium was probably confined to the surface and the glandular spaces.

In the absence of serial section, however, the 36 cases, plus the 1 "borderline" case remaining even after serial section, were still designated in the final series of cases as carcinoma in situ with gland involvement and questionable "early" stromal invasion. In the other histologic categories in the final series, there were 20 cases of carcinoma in situ of the surface alone and 178 cases of carcinoma in situ with gland involvement, which, when added to the 37 cases above, provided a total series of 235 cases of carcinoma in situ at the completion of our review.

This group of 235 cases plus the 8 in which invasive foci were found furnish an answer to a corollary of the first question, namely, "What percentage of *all* cases of carcinoma in situ, not just the 'borderline' ones, is actually invasive carcinoma?" Although 32% (8 of 25 cases) of our cases of carcinoma in situ with gland involvement and *questionable "early" stromal invasion* actually have invasive foci, only slightly more than 3% (8 of 243 cases) of *all* of our cases of carcinoma in situ had to be reclassified as invasive.

The second question posed in the introduction concerned the relative prognosis for cases in the three categories of (*a*) carcinoma in situ with gland involvement, (*b*) carcinoma in situ with gland involvement and questionable "early" stromal invasion, and (*c*) definite, but only focally invasive, carcinoma. Our data suggest that the prognosis for cases in all three groups should be about the same and very favorable when treated by simple hysterectomy, including a wide vaginal cuff. This prognostication, however, holds only when this diagnosis is based on examination of cervices which have been completely blocked or on examination of biopsy specimens from cases in which subsequent thorough further studies show no invasive carcinoma.

Thus, when a focus of questionable "early" stromal invasion is found in the routine section from one block of a completely blocked cervix, we know that if the lesion actually represents invasive carcinoma, it does not exceed the thickness of the block, i. e., 2 to 5 mm. If it were more extensive it should, of course, be apparent in the routine sections from the adjacent blocks. Similarly when a focus of questionable "early" stromal invasion is found in a biopsy specimen and no other evidence of invasion is found elsewhere, we know that the size of the lesion cannot exceed that of the biopsy specimen.

Although in our series the five-year disease-free survival rate for all three groups was 100%, the fact that 32% of cases of carcinoma in situ with gland involvement and *questionable* "early" stromal invasion were found to be actually invasive on serial section suggests that cases in this category should be given a slightly less favorable prognosis than cases of gland involvement alone. Similarly, the group of cases with definite but microscopic foci of invasion will in all likelihood have a slightly worse prognosis than the previous two groups. Thus, with increasing degrees of stromal invasion the chances in favor of lymphatic or blood vessel invasion increase and the prognosis should become less favorable. If one presupposes that any tumor cells in the stroma are capable of entering lymphatic or blood vessels, it seems reasonable to assume that sooner or later a patient with a *post-hysterectomy* diagnosis of carcinoma in situ with gland involvement and *questionable "early" stromal invasion* will develop a metastasis, due to lymphatic or capillary invasion, in an area where there was actual but not *histologically demonstrable* stromal invasion. Similarly, in cases with the histologic diagnosis of definite but minimal stromal invasion such as those just described, and those noted by Stoddard, it is almost inevitable that sooner or later some metastatic lesions will appear in an occasional patient following hysterectomy or in regional lymph nodes obtained at the time of hysterectomy and pelvic lymphadenectomy. Indeed, after this portion of the study was completed, a case of this type was reported by Decker,[6] in which a focus of metastatic tumor was found in one lymph node in a case of carcinoma in situ *with associated focal stromal invasion.*

With regard to the third question, the point at which more radical therapy should be employed, our data indicate that total hysterectomy is adequate treatment for carcinoma in situ with gland involvement and suggest that it probably would also be adequate for the other two groups as well. In those cases where the diagnosis of questionable "early" stromal invasion is made on biopsy specimens, and invasive carcinoma cannot be definitely diagnosed either

in those specimens or in adequate additional material, total hysterectomy would appear to be the treatment of choice. On the other hand, in those cases where microscopic foci of definite stromal invasion are found, we feel that each case should be treated individually and viewed as a separate therapeutic problem. Certainly here is an instance wherein the clinician must consider the available facts and decide if the mortality and morbidity in such cases treated by total hysterectomy are greater or less than the mortality and morbidity of such cases treated by Wertheim hysterectomy with pelvic lymphadenectomy or by irradiation. As indicated by the outcome of cases of microcarcinoma treated at the FHW, and the results of a study at the Pondville Hospital of cases of invasive cervical carcinoma of minimal extent treated by Wertheim hysterectomy * it would seem that the morbidity associated with any therapy is a most important factor to be considered when a choice of therapy is made. Therefore, in reference to the last question, we do not feel that we can yet designate the point on the histological spectrum between in situ and invasive carcinoma at which a really standardized form of irradiation or surgical therapy should be arbitrarily applied.

Summary

In a series of 243 cases of carcinoma in situ studied at the Free Hospital for Women, the review diagnosis in 61 cases was carcinoma in situ with gland involvement and questionable "early" stromal invasion. Semiserial sections were studied from selected paraffin blocks in 25 of these 61 cases, and microscopic foci of invasion were found in 8 cases, representing slightly over 3% of all the cases of carcinoma in situ in our series.

Certain recurrent histologic patterns of early stromal invasion are described and illustrated, and the prognostic significance of such findings is discussed. Total hysterectomy appeared to be adequate therapy for the "borderline" cases in this series, as well as for cases of carcinoma in situ without questionable "early" stromal invasion, and may very well be adequate therapy in cases of focally invasive carcinoma of minimal histologic extent.

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Immunohistochemical Analysis of Lesions Associated with "Fibrinoid Change"

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Introduction

A group of poorly understood human diseases affecting primarily the connective tissues include, among others, rheumatic fever, systemic lupus erythematosus, thrombotic thrombocytopenic purpura, and entities associated with bilateral cortical necrosis. While these diseases differ from one another in many respects, they share a histologic similarity, namely, "fibrinoid change" [1] in the lesions. Since the early studies of this change,[2,3] different, often contradictory, interpretations have been given to its nature and significance. The question of whether fibrinoid represents an intrinsic change of tissue components, either collagen,[2,4,5] ground substance,[6-8] or muscle,[9] or whether it represents a tissue change resulting from the abnormal presence of a plasma protein [10-12] or nucleoproteins [13,14] has not as yet been settled. Neither has it been settled whether all fibrinoid is essentially similar or whether diverse tissue changes of different constitution might have the typical, albeit nonspecific, staining qualities of fibrinoid. In regard to its significance, the fibrinoid change has been stressed by some authors who favor a hypersensitivity origin of some of these diseases on the basis of their morphologic similarity to experimental serum sickness, a generally accepted immunologic disorder.[6,15-19] Others have likened most of these entities to the generalized Shwartzman phenomenon, largely on the basis of the fibrinoid change, and have proposed a unifying name, "systemic fibrinoid diseases," for the group.[10] If some or all of these diseases have a pathogenesis similar to the Shwartzman phenomenon, their classification as hypersensitivity diseases would be doubtful, since most available information suggests that the Shwartzman phenomenon is not a hypersensitivity reaction in the usual immunologic or antigen-antibody sense. Recently Gitlin and co-workers [20] reported on a histochemical study of fibrinoid in the lesions of "collagen diseases," and from their results it is claimed that fibrin is an important component of such fibrinoid material. To some extent there seems to be a discrepancy between their reported findings and the results obtained in similar diseases by Vazquez and Dixon [12] and by Mellors and co-workers,[21,22] using similar techniques.

The present study was undertaken in the hope that a further analysis of the lesions of the above human diseases, plus some experimental disorders, for their plasma protein composition might indicate some similarities or differences among these various entities in which fibrinoid change is present. For this purpose, using the fluorescent antibody technique of Coons et al.,[23] the lesions of rheumatic fever, systemic lupus erythematosus, experimental serum sickness, thrombotic thrombocytopenic purpura, abruptio placenta complicated by bilateral cortical necrosis, and the generalized Shwartzman phenomenon were analyzed for their fibrinogen and/or fibrin, γ-globulin, and albumin content. From the results obtained it was possible to separate

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these entities into two immunochemically different groups: (*a*) diseases whose lesions show a preferential concentration of γ-globulin, including systemic lupus erythematosus, rheumatic fever, and experimental serum sickness, and (*b*) diseases whose lesions show a preferential concentration of fibrinogen and/or fibrin, namely, thrombotic thrombocytopenic purpura, abruptio placenta with bilateral cortical necrosis, and the generalized Shwartzman phenomenon. Additional studies were done on the fibrinoid change in peptic ulcer and in human placenta which showed that in such lesions there is a preferential concentration of fibrinogen and/or fibrin.

Material and Methods

The different antisera used for conjugation to fluorescein were obtained by animal immunizations with different fractionated plasma protein antigens. The antihuman γ-globulin (anti-HGG), antihuman serum albumin (anti-HSA), antirabbit γ-globulin (anti-RGG), and antirabbit serum albumin (anti-RSA) used in this experiment were the same as previously described.[24] Antihuman fibrinogen (anti-HFib) was obtained after giving albino rabbits averaging 3 kg. in body weight injections of fractionated soluble human fibrinogen.* The antigen was injected according to the following schedule: On Days 1 and 2 the animals received 10 mg. subcutaneously. On Days 3 and 4 the dose was increased to 20 mg. by the same route, and on Day 5 each animal received 30 mg. per kilogram intravenously. The same course was given after two weeks and repeated thereafter in a similar fashion, as needed. The serum obtained contained approximately 400 to 500μg. of antibody nitrogen per milliliter. Antirabbit fibrin (anti-RFib) was obtained after giving an injection to a sheep of an insoluble preparation of rabbit fibrin obtained as follows: Heparinized blood was drawn from normal rabbits, and after separation of cells the plasma was whipped and thrombin was added. Fibrin threads were collected and washed repeatedly with saline. The resultant insoluble white material was then lyophilized and reduced to a fine powder. The fibrin thus obtained was emulsified in Freund's adjuvant (without Mycobacterium tuberculosis bacilli), and the animal was given injections according to the following schedule: An initial intramuscular dose of 30 mg. of protein was given, and after approximately three weeks, 50 mg. was given by the same route. This last dose was repeated thereafter at three- to four-week intervals. By the fifth injection and subsequently, the sheep sera contained approximately 500μg. of antibody nitrogen per milliliter. Both antisera (anti-HFib and anti-RFib) were immunologically characterized by precipitin reactions in saline and in gel media by Dr. Paul H. Maurer. It was found that at the various concentrations tested, no detectable cross reaction occurred between anti-RFib and RGG or RSA. Similarly, no cross reaction was found between anti-HFib and HGG or HSA. The antifibrinogen antisera were not pure, however, as they showed some cross reaction with α- and β-globulins with the precipitin techniques employed. These antisera could be further purified by absorption with the corresponding normal serum (fibrinogen free) at optimal proportions. In this work the terms fibrinogen and fibrin, when used to describe a histochemical component, are interchangeable, as immunologic cross reaction occurs between antifibrinogen and fibrin, and vice versa. All antisera thus obtained were conjugated to fluorescein isocyanate † as previously described,[23,24] and the resulting fluorescent antibody conjugates were used as specific histochemical stains for the detection of the corresponding antigen in frozen tissue sections. The techniques employed for the purification of conjugates, staining of sections, fluorescence microscopy, and photography were similar to those described elsewhere.[23,24] Likewise, the scheme of technical controls for the specificity of fluorescence was employed as previously described.[24] A modification of the staining procedure was concurrently used in this experiment to offer further control on the specificity of the antisera. Thus, when a section was stained with fluorescent anti-γ-globulin for the detection of γ-globulin in tissue, an adjacent similar section was treated with the same reagent after it had been exposed previously to nonfluorescent antifibrinogen for 30 minutes. This was done in order to block fibrinogen (at the slide level) immunologically, if present in the section, which might react with the fluorescent anti-γ-globulin in case this conjugate were to contain some fluorescent antifibrinogen. However, no evidence for the presence of fluorescent antifibrinogen was seen in fluorescent anti-γ-globulin conjugates. Similarly, and on the same basis, when fluorescent antifibrinogen was utilized to detect fibrinogen in tissues, an adjacent section was exposed to nonfluorescent anti-γ-globulin prior to specific staining.

All tissues employed were obtained either at autopsy or by biopsy. Small representative blocks for immunohistochemical studies were quickly frozen (at —70 C) and stored in deep freeze

* Sharp & Dohme, Division of Merck & Co., Inc., Lot No. 31289-31.

† Fluorescein amine was obtained from Dr. Klaus Hofmann, Department of Biochemistry, University of Pittsburgh School of Medicine.

(at —30 C) until ready for sectioning. Comparable blocks were also fixed in 10% formaldehyde for further morphologic studies. Sections were routinely stained with hematoxylin and eosin, periodic acid-Schiff (PAS) stain, and toluidine blue.

For convenience of description, the different tissues employed in this study will be described under separate headings according to disease.

Rheumatic Fever.—Material used included hearts from three cases of rheumatic carditis in children. Activity in the disease was evidenced by clinical studies and postmortem examination. Morphologic studies of the myocardium showed increased edematous perivascular connective tissue in addition to "Aschoff bodies" (Fig. 1). Acute pericarditis was present with moderate cellular infiltration and fibrinous deposition. An auricular appendage of an additional case of active chronic rheumatic carditis was studied.‡ This material showed no typical Aschoff bodies, only edema of subendocar-

‡ The material in this case was sent to us by Drs. M. Laufe and J. Rigler, University Clinics, The University of Chicago.

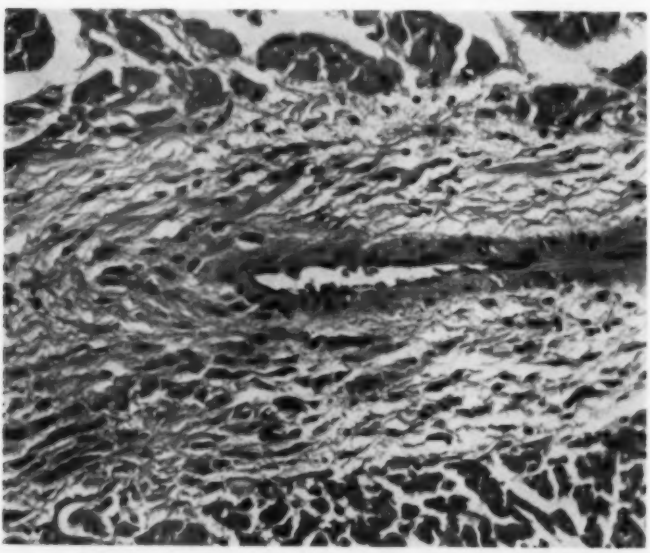

Fig. 1.—Section of human myocardium in a case of active rheumatic carditis. Note increased perivascular connective tissue infiltrated with mononuclear inflammatory cells. Hematoxylin and eosin stain.

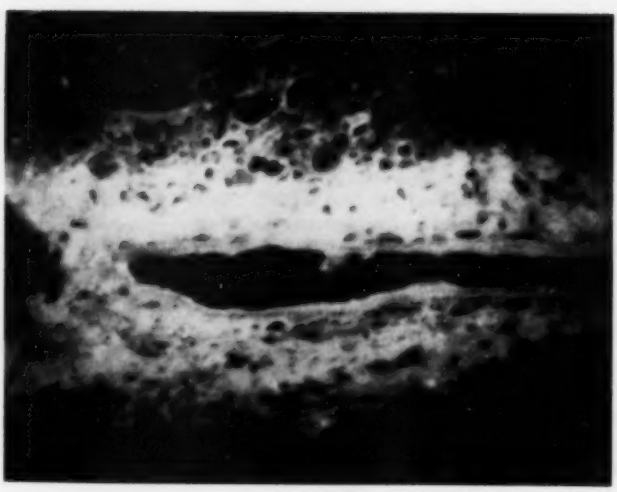

Fig. 2.—Section of myocardium in active rheumatic carditis, same case as in Figure 1, stained with fluorescent anti-HGG. Note the diffuse specific fluorescence in the inflamed perivascular connective tissue, indicating concentrations of γ-globulin in these areas. Fluorescent micrograph.

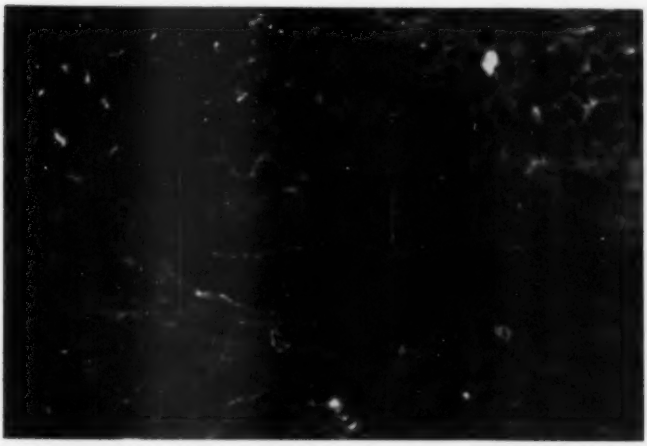

Fig. 3.—Section of myocardium immediately adjacent to the section in Figure 2 stained with fluorescent anti-HFib. Note the absence of specific fluorescence in the perivascular connective tissue when compared to that in Figure 2, indicating lack of concentrations of fibrinogen in these areas. Fluorescent micrograph.

dial connective tissue and fibrinous deposition on the endocardial surface (Fig. 4).

Systemic Lupus Erythematosus.—Material used included the kidneys in three cases of systemic lupus erythematosus (SLE) in which clinical evidence of positive L. E.-cell phenomenon was present. Morphologically there were characteristic changes as described in SLE, such as "wire loop" appearance of glomerular capillaries with thickening of so-called basement membrane by fibrinoid. Prominent fibrinoid change in renal arterioles was also present (Figs. 7, 15).

Thrombotic Thrombocytopenic Purpura and Abruptio Placenta with Bilateral Cortical Necrosis.—Three cases of thrombotic thrombocytopenic purpura (two children and one adult) were studied. The lungs, kidneys, pancreas, liver, and spleen in these cases showed morphologic evidence of generalized small vessel occlusion by material with a homogeneous eosinophilic character. Some vessel walls showed an eosinophilic change resembling fibrinoid without occlusion of their lumens. The glomerular capillaries also showed fibrinoid (Figs. 9, 18, 21). The kidneys, lung, and liver in one case

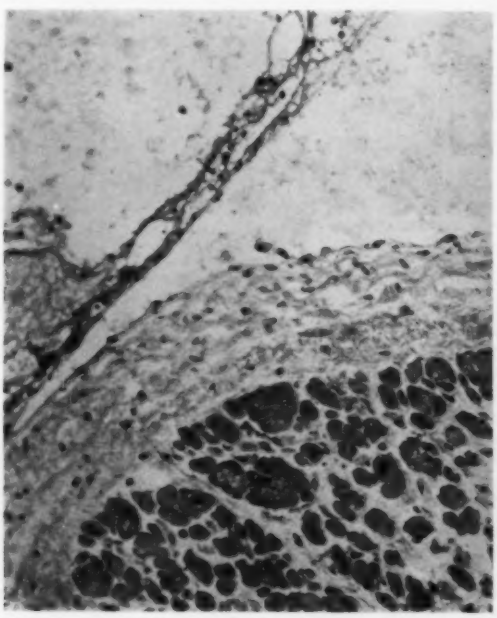

Fig. 4.—Section of human auricular appendage in a case of chronic rheumatic carditis, showing an edematous endocardial layer with fibrinous deposition in the surface. Hematoxylin and eosin stain.

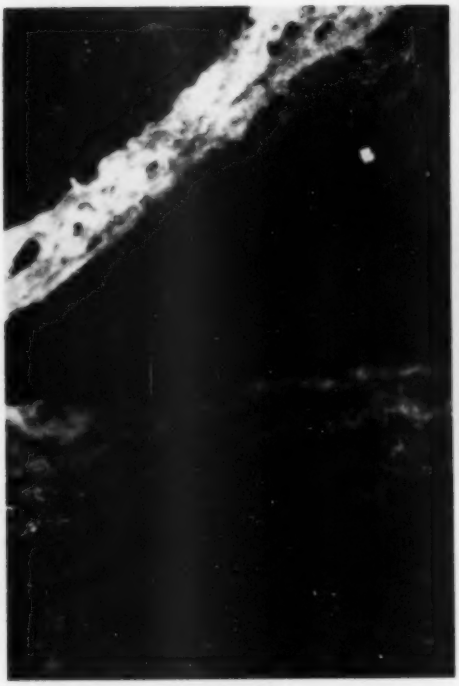

Figure 5

Fig. 5.—Section of auricular appendage in chronic rheumatic carditis similar to that in Figure 4, stained with fluorescent anti-HFib.

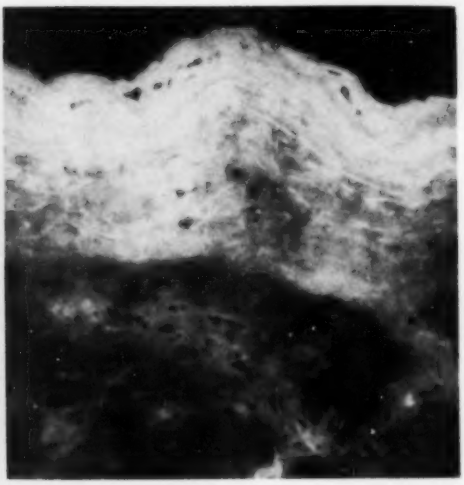

Figure 6

Note the bright specific fluorescence of the fibrinous material above the endocardial surface, indicating concentrations of fibrinogen and/or fibrin in such material. By contrast, the edematous endocardial layer shows lack of concentration of fibrinogen. Fluorescent micrograph.

Fig. 6.—A higher magnification of a section similar to that in Figure 5, stained with fluorescent anti-HGG. Note, in contrast to Figure 5, the bright specific fluorescence in the edematous endocardial layer, indicating concentration of γ-globulin in this area. Fluorescent micrograph.

Fig. 7.—Section of human kidney in a case of systemic lupus erythematosus, showing a glomerulus with prominent fibrinoid change in wall of capillaries in a "wire loop" fashion. Hematoxylin and eosin stain.

Fig. 8.—Section of kidney in systemic lupus erythematosus similar to that shown in Figure 7, stained with fluorescent anti-HGG. Note the bright green specific fluorescence of the thickened capillary walls, indicating concentrations of γ-globulin in the fibrinoid. Fluorescent micrograph.

Fig. 9.—Arteriole in a case of thrombotic thrombocytopenic purpura, showing fibrinoid material partially occluding the lumen and incorporated in the vessel wall. Hematoxyin and eosin stain.

Fig. 10.—An arteriole in thrombotic thrombocytopenic purpura similar to that seen in Figure 9, stained with fluorescent anti-HFib. Note the bright green specific fluorescence, indicating concentration of fibrinogen and/or fibrin in the fibrinoid partially occluding the lumen and incorporated in the vessel wall. Fluorescent micrograph.

Fig. 11.—Section of rabbit kidney in the generalized Shwartzman phenomenon, showing a glomerulus with fibrinoid material occluding the capillary lumens. Hematoxylin and eosin stain.

Fig. 12.—Section of rabbit kidney in the generalized Shwartzman phenomenon similar to that seen in Figure 11, stained with fluorescent anti-RFib. Note the green specific fluorescence, indicating concentrations of homologous "fibrinogen" in the fibrinoid occluding the capillaries. Fluorescent micrograph.

Fig. 13.—Section of human placenta showing chorionic villus with fibrinoid. Hematoxylin and eosin stain.

Fig. 14.—A section of human placenta similar to that seen in Figure 13, stained with fluorescent anti-HFib. Note the bright green specific fluorescence, indicating concentrations of fibrinogen and/or fibrin in the fibrinoid change. Fluorescent micrograph.

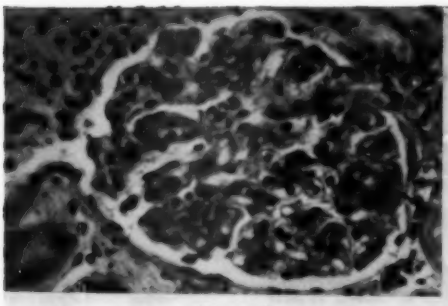

Figure 7

Figure 8

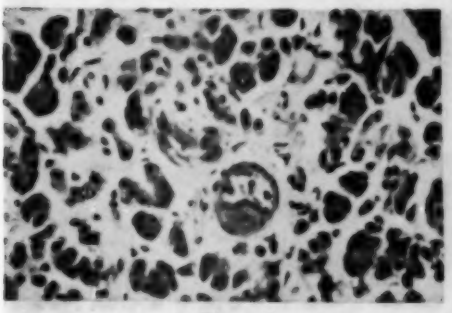

Figure 9

Figure 10

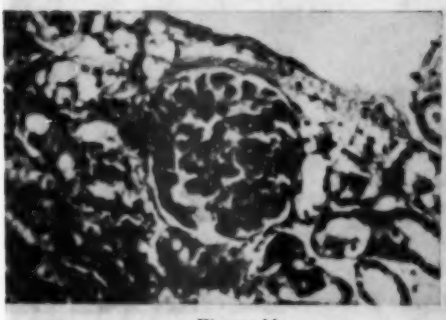

Figure 11

Figure 12

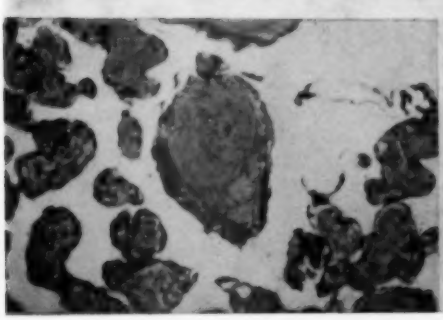

Figure 13

Figure 14

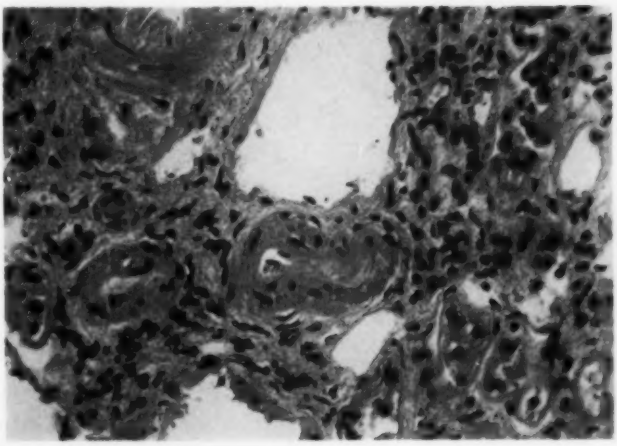

Fig. 15.—Section of human kidney in a case of systemic lupus erythematosus showing arterioles with fibrinoid change. Hematoxylin and eosin stain.

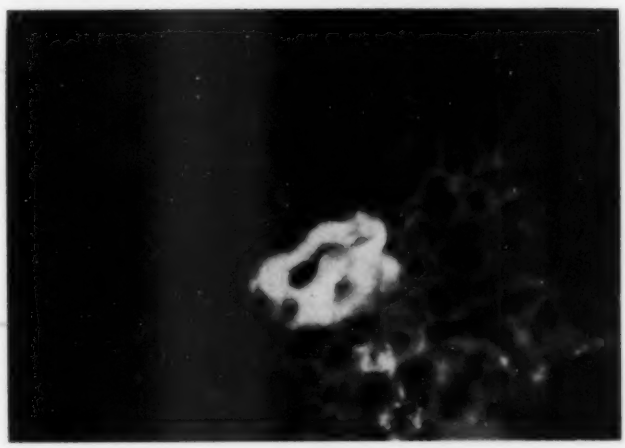

Fig. 16.—Section of human kidney in systemic lupus erythematosus similar to that seen in Figure 15, stained with fluorescent anti-HGG. Note the bright specific fluorescence in vessel wall, indicating concentrations of γ-globulin in the fibrinoid. Fluorescent micrograph.

Fig. 17.—Section of kidney in systemic lupus erythematosus similar to that seen in Figure 16, stained with fluorescent anti-HFib. Note, by contrast, the absence of specific fluorescence in the altered vessel wall, indicating lack of concentrations of fibrinogen. Fluorescent micrograph.

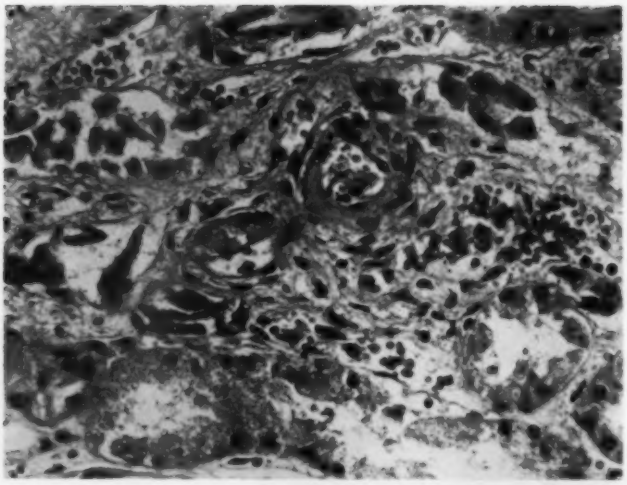

Fig. 18.—Section of kidney in a case of thrombotic thrombocytopenic purpura with fibrinoid change in arterioles. Hematoxylin and eosin stain.

Fig. 19.—section of kidney in thrombotic thrombocytopenic purpura similar to that seen in Figure 18, stained with fluorescent anti-HFib. Note the bright specific fluorescence, indicating concentrations of fibrinogen in the fibrinoid of arterioles. Fluorescent micrograph.

Fig. 20.—A section of kidney similar to that seen in Figure 19, stained with fluorescent anti-HGG. Note, by contrast with Fig. 19, the absence of specific fluorescence in the fibrinoid of arterioles, indicating lack of concentration of γ-globulin in these altered vessels. Fluorescent micrograph.

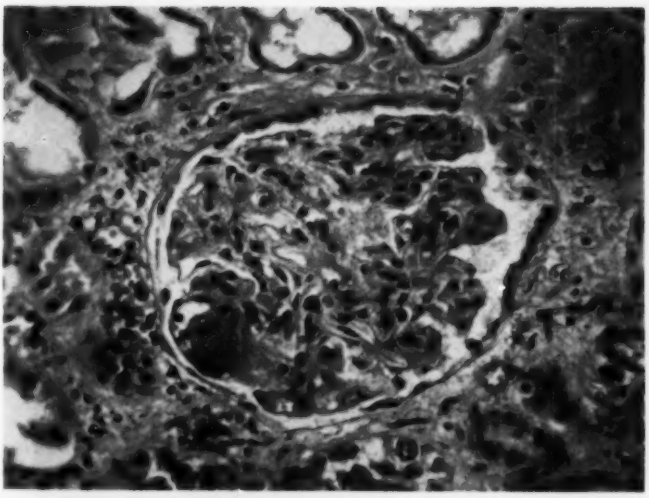

Fig. 21. — Section of human kidney in thrombotic thrombocytopenic purpura to show a glomerulus with prominent fibrinoid change. In one area there is a suggestion of partial occlusion of a dilated capillary lumen by fibrinoid. Hematoxylin and eosin stain.

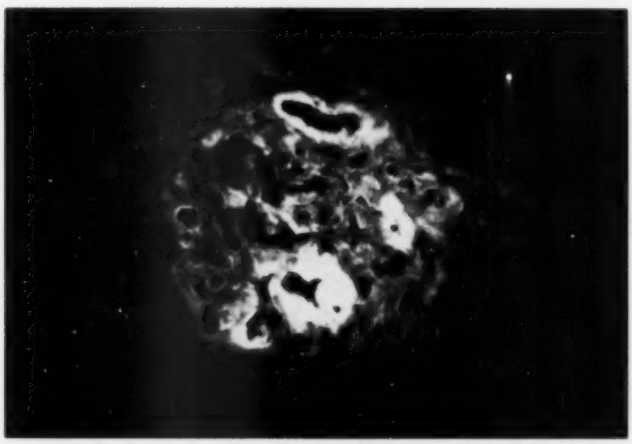

Fig. 22.—Section of human kidney in thrombotic thrombocytopenic purpura similar to that in Figure 21, stained with fluorescent anti-HFib. Note the bright specific fluorescence of the fibrinoid in the capillaries, indicating concentration of fibrinogen in these areas. Fluorescent micrograph.

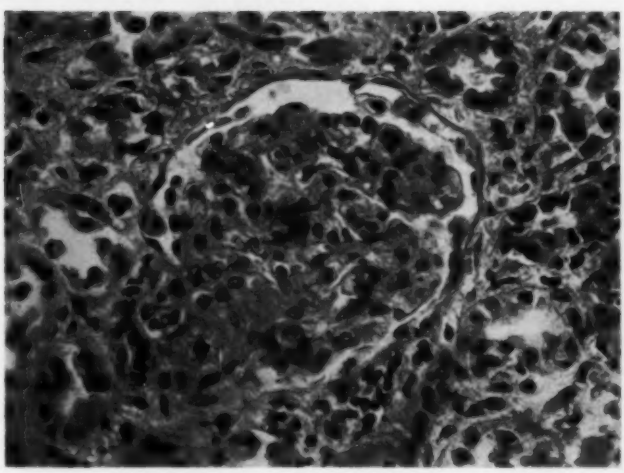

Fig. 23.—Section of human kidney in a case of abruptio placenta complicated by bilateral cortical necrosis. Prominent fibrinoid change is noted in the glomerulus. Hematoxylin and eosin stain.

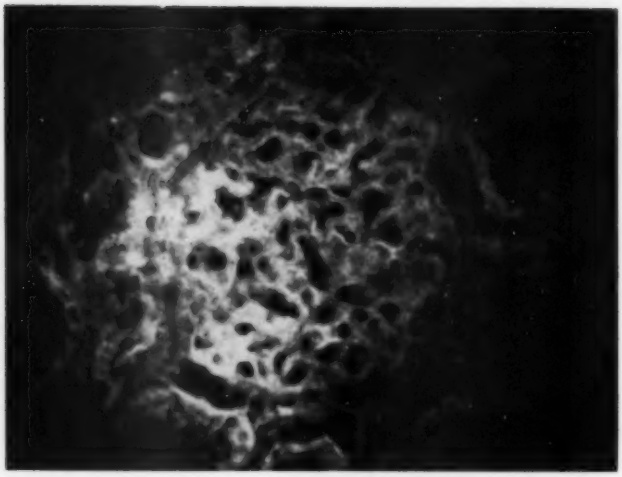

Fig. 24.—A section of kidney similar to that seen in Figure 23, stained with fluorescent anti-HFib. Note the bright specific fluorescence, indicating concentrations of fibrinogen in the areas of fibrinoid change. Fluorescent micrograph.

of premature separation of the placenta complicated by bilateral cortical necrosis were studied, and this case is included along with the cases previously described of thrombotic thrombocytopenic purpura because of their similar morphologic changes. This case showed generalized vascular occlusive phenomena with eosinophilic material resembling fibrinoid; the kidneys were the seat of bilateral cortical necrosis, and fibrinoid change was present in arterioles and glomeruli (Fig. 23).

Experimental Serum Sickness.—Serum sickness was produced in rabbits by a single intravenous injection of radiolabeled crystalline bovine serum albumin (I^{131} BSA), 250 mg. per kilogram. The rate of disappearance of injected antigen was followed by radioisotope tracer techniques,[20] and the animals were killed on the day of complete immune antigen elimination, usually 13 to 15 days after injection of antigen. This group of animals showed lesions similar to those described previously,[18,19] consisting mainly of a proliferative glomerulitis and coronary vessel wall damage with endothelial proliferation.

Generalized Shwartzman Phenomenon.—The generalized Shwartzman reaction was provoked in albino rabbits weighing approximately 1.5 to 2 kg. by a preparatory intravenous dose of 75μg. of Escherichia coli endotoxin,§ followed 24 hours later by a similar intravenous provoking dose of E. coli endotoxin. The animals were killed at intervals up to 24 hours after the second dose. Histologic evidence of generalized vascular occlusive phenomena was present. The occlusive material consisted of clumps of smudgy eosinophilic material, sometimes admixed with blood cells, mainly leukocytes. In some of the animals the eosinophilic occlusive material was plastered against the vessel walls and endothelium was covering this material, with the resulting incorporation of fibrinoid in the vessel wall. The kidneys showed bilateral cortical necrosis as early as four hours after the provoking dose, and histologically many glomeruli showed plugging of their capillary lumens with eosinophilic material (Fig. 11).

Miscellaneous Conditions.—One case of active gastric peptic ulcer was studied in which fibrinoid change at the base of the lesion was present. Placental tissues with prominent fibrinoid change were also studied (Fig. 13).

§ Lipopolysaccharide E. coli, O111:B_4, Difco Laboratories R_a B35527.

Results

Rheumatic Fever.—When areas of myocardium with active lesions, as previously described, were stained with fluorescent anti-HGG, diffuse intense specific fluorescence was observed in the edematous perivascular connective tissue, indicating the presence of considerable amounts of homologous γ-globulin in these areas. Because of the diffuse distribution of γ-globulin in the altered perivascular connective tissue, the visual localization of increased concentrations of this protein in the specific areas of fibrinoid was difficult (Fig. 2). When similar sections were stained for albumin, no significant concentrations of this protein were found in the altered perivascular connective tissue. These findings are comparable to those previously described.[12]

Similarly, when sections were stained with fluorescent anti-HFib, no specific fluorescence was detected, indicating the absence of concentrations of fibrinogen in these areas (Fig. 3). However, when sections of acutely inflamed epicardium were immunohistochemically stained for γ-globulin, fibrinogen, and albumin, all of these three proteins were present, fibrinogen (or fibrin) being particularly concentrated in or near surfaces, usually with a fibrillar character. This pattern of protein distribution might be expected in areas of acute fibrinous inflammation with outpouring of all plasma proteins. When sections taken from the auricular appendage in a case of active rheumatic carditis were stained with fluorescent anti-HGG, the edematous endocardial layer showed preferential concentrations of homologous γ-globulin (Fig. 6). When similar sections were stained for albumin or fibrinogen, these areas showed no concentrations of these proteins. It is of interest that in some areas there was deposition of eosinophilic fibrillar material in the surface of endocardium, which had the morphologic appearance of fibrin threads. When sections showing this feature were stained for γ-globulin, the endocardium again showed concentrations of γ-globulin, whereas the deposited fibrillar material was negative. On the other hand, when these areas were stained for fibrinogen (or fibrin) the fibrillar material on the surface was positive, whereas the edematous endocardium was negative (Fig. 5).

Systemic Lupus Erythematosus.—When sections of kidneys in these cases were stained with fluorescent anti-HGG, the fibrinoid change of arterioles and capillary walls of glomeruli showed specific concentrations of γ-globulin (Figs. 8 and 16), a finding similar to that previously reported.[12] When similar sections were stained with fluorescent anti-HFib or fluorescent anti-HSA for the detection of fibrinogen and albumin, respectively, no significant specific fluorescence was noted, indicating lack of concentration of these proteins in the fibrinoid (Fig. 17).

Thrombotic Thrombocytopenic Purpura and Abruptio Placentae.—When sections of kidneys, liver, pancreas, spleen, and lungs in cases of thrombotic thrombocytopenic purpura were stained with fluorescent anti-HFib, specific concentrations of fibrinogen were found in the occlusive material of small vessels. Sometimes this material was observed occluding completely or partially the vessel lumens; at other times it was seen as forming part of the vessel wall. In either case, fibrinogen was found in this material (Figs. 10 and 19). When similar lesions were studied for albumin or γ-globulin, no concentrations of these proteins were found (Fig. 20). In sections of kidneys with fibrinoid change in glomerular capillaries, specific concentrations of fibrinogen were found in these areas, whereas no concentrations of albumin or γ-globulin were present (Fig. 22). Findings similar to those described for thrombotic thrombocytopenic purpura were found in the occlusive material and vessel walls in one case of abruptio placentae complicated by bilateral cortical necrosis (Fig. 24).

Experimental Serum Sickness.—Most of the immunohistochemical data in experimental serum sickness has been previously reported.[19] Evidence was given for the presence of specific concentrations of homologous γ-globulin in glomeruli showing a proliferative glomerulitis, as well as in the wall of damaged coronary vessels (arteritis). Likewise, evidence was presented for the localization of the specific antigen and, indirectly, the antibody in such lesions. When sections of kidneys with glomerulitis were stained with fluorescent anti-RFib, no specific concentration of fibrinogen was found. Similar sections showed concentrations of γ-globulin in altered glomeruli when stained with fluorescent anti-RGG.

Generalized Shwartzman Phenomenon.—When sections of kidneys, liver, lungs, and spleen of rabbits showing the generalized Shwartzman reaction were stained with fluorescent anti-RFib, it was seen that the occlusive material in small vessels of dif-

ferent organs as well as the fibrinoid material in glomeruli contained specific concentrations of homologous fibrinogen (Fig. 12), as previously reported.[26,27] No concentrations of γ-globulin or albumin were found in these areas.

Miscellaneous Conditions.—The fibrinoid change in the base of peptic ulcer showed specific concentrations of homologous fibrinogen (or fibrin) when the sections were stained with fluorescent anti-HFib. The areas of specific fluorescence were localized to the "fibrinoid strands" in the base of the ulcer. When similar sections were stained with fluorescent anti-HGG and fluorescent anti-HSA, diffuse increases of both γ-globulin and albumin were found in these areas, distributed much more widely in the inflammatory lesion than the fibrinogen. The fibrinoid in placental tissue stained specifically for fibrinogen (Fig. 14). γ-Globulin and albumin were found in the connective tissue stroma of the chorionic villi, a finding similar to that recently reported by Bardawil et al.[28]

Comments and Conclusions

Immunohistochemical methods such as the fluorescent antibody technique appear to be better equipped to obtain information regarding the presence of some specific integral substance in tissues than other available methods, morphologic, histochemical, or enzymatic, or a combination of these. While the identification of tissue substances with the fluorescent antibody technique enjoys the specificity of an immunologic reaction, little or no quantitative information can be derived from such observations. Only relative ideas of concentrations of a given substance can be obtained by comparing the intensity of fluorescence in one area of a tissue section to another area of the same section. However, by comparing the intensity of the stain for a given substance in both normal and diseased tissues, marked differences can be observed which presumably reflect differences in concentrations of the specific substance. Thus, using adjacent sections stained for fibrinogen, γ-globulin, and albumin, respectively, it may be possible to recognize a relative concentration of one or more of these proteins in a given lesion above the concentration in normal areas, while other proteins may not show a similar concentration in the lesion.

Although the finding of specific plasma protein or proteins in a given lesion with fibrinoid does not necessarily account for the whole amount and composition of this material, it does focus attention on a component that might be instrumental in the genesis of the fibrinoid. The findings of concentrations of γ-globulin in lesions of rheumatic fever and systemic lupus erythematosus are in agreement with previous observations made by us [12] and by Mellors et al.[22] on these diseases and on rheumatoid arthritis and polyarteritis nodosa. However, they fail to confirm the findings of Gitlin and co-workers [20] in these diseases, using similar procedures. The technical steps employed by the latter authors are somewhat different from the ones we have employed in this study, and some of their morphologic observations bear little resemblance to the ones reported here.

While our results serve to divide the entities studied into two groups based on the plasma protein composition of the lesions, they do little to explain the pathogenic mechanisms involved. The concentration of γ-globulin in certain lesions has been cited as evidence for the operation of an antigen-antibody mechanism.[21,22] Certainly, a localized antigen-antibody reaction, such as must occur in experimental serum sickness, can be responsible for a localization of γ-globulin in a lesion. Likewise, the finding of preferential concentrations of γ-globulin in the lesions of systemic lupus erythematosus is consistent with the direct participation of the L. E. factor, a γ-globulin, in the pathogenesis of the lesions. However, it is possible that γ-globulin concentrations in primary connective tissue changes might also be due to increased affinity of that

tissue for γ-globulin. It is known that there is normally a small affinity of γ-globulin for some tissue constituents, and this might be magnified in such situations. It is also possible that a metabolic alteration leading to hypergammaglobulinemia, such as is frequently found in some of these diseases, might predispose to the deposition of γ-globulin in either normal or abnormal tissues.

In contrast to the lesions showing concentrations of γ-globulin, the lesions of the generalized Shwartzman reaction, thrombotic thrombocytopenic purpura, and bilateral cortical necrosis associated with premature separation of the placenta show preferential concentrations of fibrinogen. These lesions consist largely of intravascular fibrinoid which may become incorporated into the vessel walls. The fibrin nature of these "thrombi" has been suggested, primarily on the basis of the fall in plasma fibrinogen levels at the time these thrombi are formed [29,30] and as result of electron microscope studies.[31] Our findings are consistent with these suggestions and confirm previous observations on the nature of the fibrinoid material in thrombotic thrombocytopenic purpura made by Craig and Gitlin,[32] using immunohistochemical techniques. Whether the deposition of fibrinogen is primary or whether it is preceded by a change in the vessel wall cannot be determined, but it seems likely that the fibrinoid material in the vessel walls and thrombi represents fibrinogen deposited from the circulation, and is not primarily composed of altered constituents of the vessel wall, as has been suggested.[33,34] The similarity of the immunohistochemical findings in the generalized Shwartzman phenomenon and bilateral cortical necrosis associated with premature separation of the placenta are consistent with previous observations [35] on the resemblance of these two entities. The morphologic similarity of lesions showing increased fibrinogen concentrations might suggest a similar set of steps in the development of the vascular lesions, i. e., deposition of fibrinogen in vessel walls and thrombosis. However, a variety of predisposing and precipitating factors might lead to this final event. For instance, the initial injury might be to the vessel itself or to the circulating elements involved in coagulation or to both. In the generalized Shwartzman phenomenon, both these factors appear to play a role, but much less is known of the pathogenesis of the human diseases.

It may be concluded (*a*) that the results of an immunohistochemical analysis of lesions associated with fibrinoid change are consistent with the views that such change is not chemically identical in all cases [36,37]; (*b*) that plasma proteins might play a role in the production of the fibrinoid change, and (*c*) that, in our present state of ignorance regarding the so-called connective tissue diseases, results such as these presented can have little pathogenetic significance. These findings can only serve to point out differences and, perhaps less surely, similarities between some of the diseases studied. The observations, however, emphasize the danger in applying a unifying concept to fibrinoid and to the diseases associated with this change.

Miss Judith Carnahan and Mr. William Arnold gave technical assistance and photography was by Mr. Robert Laughlin and staff. The human materials for this study were provided by Dr. T. J. Moran and staff of the Department of Pathology, Presbyterian Hospital; Dr. George Fetterman and staff of the Department of Pathology, Children's Hospital of Pittsburgh, and Dr. R. G. McManus and staff of the Department of Pathology, The Western Pennsylvania Hospital.

Department of Pathology, University of Pittsburgh School of Medicine (13).

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Papillary Carcinoma of the Thyroid

A Study of the Pathology of Two Hundred Twenty-Six Cases

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Papillary carcinoma of the thyroid, the commonest type of thyroid cancer,[1,2] often has such a slow clinical course that it poses special problems in initial and long-term management.[3,4] Since clinicians often regard and treat these tumors in a manner very different from others, the pathologist's classification of a thyroid cancer as papillary is of more than academic interest. For the same reasons, a comprehensive knowledge of the pathology of these tumors is of special importance.

Material and Methods

The present study is based on 226 surgical specimens of papillary thyroid carcinoma seen in this laboratory during the 20 year period, 1937-1956. Each specimen was reviewed from two sources: (*a*) slides for microscopic study, and (*b*) laboratory records of gross and clinical data. The criteria used for diagnosing a thyroid tumor as papillary carcinoma were (*a*) formation of papillae, hence papillary, and (*b*) evidence of invasion, hence carcinoma. Furthermore, only tumors showing predominantly (over 50%) papillary structure were included in this series. The term papillary is perhaps less exact than the term papilliferous, but it is retained because of established usage.

Results

Occurrence.—As in most reported series of thyroid cancer, the majority of the patients (81%) were female.

Age distribution is shown in Figure 1. The range was 14 to 86, with the median age 42. The great majority were rather evenly distributed within the third through seventh decades.

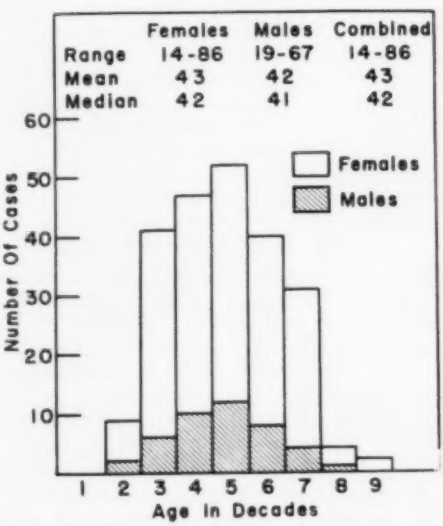

Fig. 1.—Age distribution by sex in 226 cases of papillary thyroid carcinoma.

The ratio of papillary to all thyroid cancers seen in this laboratory during the past 30 years has remained very constant. Within each decade the papillary type has consistently made up approximately 50% of the total.

Gross Appearance.—Color of the tumor tissue was typically tan, but with degenerative changes, which were common, there were foci of red (hemorrhage), gray (fibrosis), and occasionally yellow (necrosis). Consistency was resilient to firm in the more solid tumors and soft to friable in those with cyst formation. In either case there was sometimes gross evidence of focal calcification.

Cyst formation ("cystadenocarcinoma") was frequent (47%). The cavities were filled with thin brown fluid and varied in

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diameter from less than 1 cm. to as much as 3 or 4 cm.

Papillary structure was sometimes evident grossly; in the cystic tumors, as papillary projections along the inner lining of the cyst wall, and in the solid tumors, as a rough or shaggy texture of the cut surface.

A capsule was described grossly in 52 cases (22%). Much more commonly, however, the tumor margins were too ill defined for statistical evaluation. Grossly discrete tumors typically measured about 2 cm. in diameter, weighed about 10 gm., and showed no predilection for any particular portion of the gland.

From the gross examination alone, a presumptive diagnosis of papillary carcinoma can often be entertained, especially in the more discrete tumors with cysts. However, it should be emphasized that adenomatous goiter or follicular tumors may at times have a similar gross appearance.

Microscopic Appearance.—The characteristic feature of papillary growth was the arrangement of the tumor epithelium on fibrovascular stalks projecting into cystic spaces (Fig. 2). The tumor epithelium was typically arranged in single-cell layers, but at tangentially cut papillary tips it sometimes appeared heaped up or multilayered. The typical cells were cuboidal to low columnar and had homogeneous or slightly vacuolated amphophilic cytoplasm surrounding vesicular, centrally placed, round to ovoid nuclei (Fig. 3).

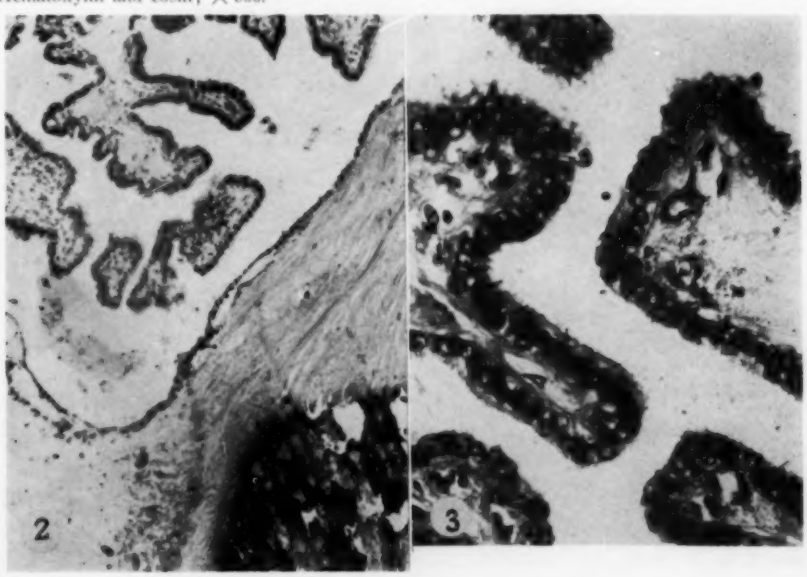
Variations in tumor pattern, itemized in Table 1, occurred as foci of follicular and/or simplex type, scattered irregularly through-

TABLE 1.—*Variation in Tumor Pattern*

	Cases	
	No.	%
Pure papillary	108	48
Focal follicular	87	39
Focal follicular & simplex	19	8
Focal simplex	12	5
	226	100

Fig. 2.—Low-power view of typical papillae. Note also stromal calcification. Hematoxylin and eosin; × 100.

Fig. 3.—High-power view of papillae. The tumor epithelium is in typical single-layer arrangement on the lower papilla and seemingly heaped up on the upper, tangentially cut, papilla. Hematoxylin and eosin; × 500.



out the predominantly papillary mass (Fig. 4). Lymph nodal metastases, when present, usually showed similar and corresponding variation in pattern of tumor growth. Follicular foci occasionally showed some degree of nuclear pleomorphism and some mitoses, but no increase in frequency of tumor invasion and metastasis per se was found in focally follicular, as contrasted with purely papillary, primary tumors. Simplex foci, here defined as tumor growth in solid cell clusters without formation of follicles or papillae, showed less cytologic pleomorphism than follicular foci and also no distinct correlation with tumor invasiveness.

Variations in tumor cytology were (*a*) clear cells (Fig. 5), similar to the large clear cells of parathyroid tissue and hypernephroid tumors, which occurred in 64 cases (28%) as small nests and as single cells admixed with more typical papillary epithelium, and (*b*) Hürthle cells (Fig. 6), found in 18 cases (8%) and usually associated with foci of predominantly follicular rather than papillary pattern. Cilia-like structures appeared along the papillary tumor epithelium in seven cases (3%). These structures differed from true cilia, however, in being irregularly scattered rather than brush-like and of variable rather than uniform thickness.

Aside from these variations in tumor pattern and cell type, none of which showed definite relation to tumor invasiveness, pleomorphism was minimal. Mitoses were very rare and even when present did not appear to correlate with tumor invasiveness.

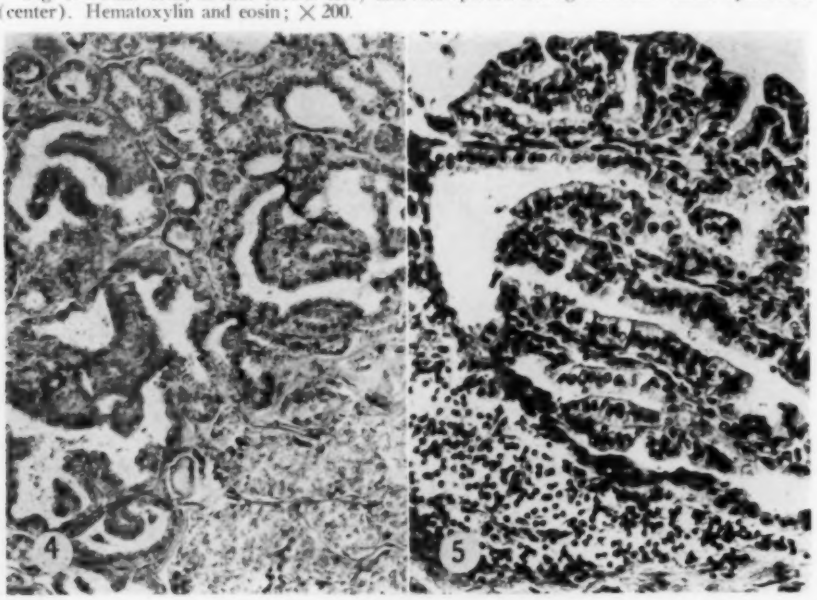
Squamous metaplasia was found in 101 of the 226 cases (45%), appearing as

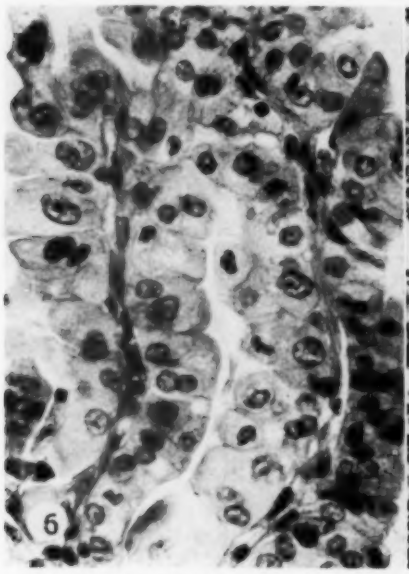
TABLE 2.—*Special Structures and Characteristics*

	Cases	
	No.	%
Squamous metaplasia	101	45
Psammoma bodies	89	39
Clear-cell foci	64	28
Stromal calcification	40	18
Hürthle-cell foci	18	8

Fig. 4.—Variations in growth pattern are papillary, follicular, simplex. Hematoxylin and eosin; × 125.

Fig. 5.—Clear cells, in nest (lower left) and interspersed among columnar tumor epithelium (center). Hematoxylin and eosin; × 200.





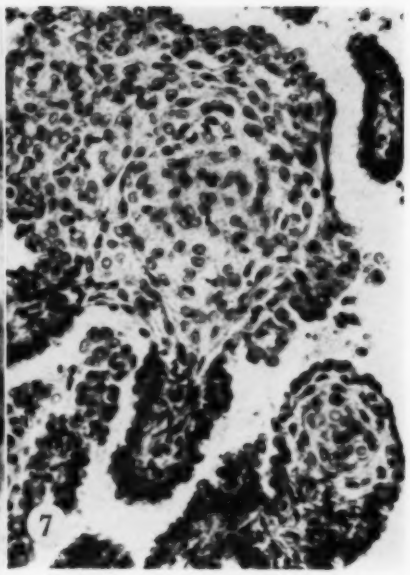


Fig. 6.—Hürthle-cell focus in papillary tumor. Hematoxylin and eosin; × 500.
Fig. 7.—Squamous metaplasia of tumor epithelium. Hematoxylin and eosin; × 200.

whorled nests of keratinized epithelium duplicating the prickle-cell and squamous layers of the epidermis but only rarely forming distinct epithelial pearls (Fig. 7). This change had no distinct topographical or quantitative relation to stromal inflammation.

Psammoma (Greek psammos, sand) bodies (Fig. 8), defined as laminated calcific spherules, were also frequent (39%) and usually concurrent with, but not topographically related to, squamous metaplasia. The origin of these psammoma bodies appeared to be from hyalinized stroma of the papillae in both primary and metastatic tumor. The spherules were also found independent of tumor, lying free in the interspaces of both marginal and distant thyroid tissue, suggesting that they may have undergone intraglandular dissemination.

Stromal changes were numerous. Hyalinizing fibrosis was almost universal but varied in degree without constant relation to tumor invasiveness or frequency of psammoma-body formation. Slight interstitial hemorrhage, a common finding, was often accompanied by lipophages and occasionally also by cholesterol clefts and foreign-body giant cells. Stromal calcification (Fig. 2) was present in 40 cases (18%) and appeared similar to the calcium deposition sometimes found within desmoplastic slow-growing tumors of other tissues. Common changes in the fibrovascular stalks of the papillae were infiltration of round cells, especially plasmocytes, and interstitial edema. Interstitial lymphocytic infiltration was frequent and sometimes formed discrete lymphoid follicles. Foci of necrosis, ischemic in type, were found in nine cases (4%). As with the interstitial fibrosis and hemorrhage, none of these cellular inflammatory changes showed distinct correlation with tumor invasiveness.

Invasion.—Invasion was of four types, as defined by Warren and Feldman[5]: lymphatic, venous, capsulostromal, and intraglandular. One or more types was present in each case (Table 3). By far the commonest route was lymphatic, present in

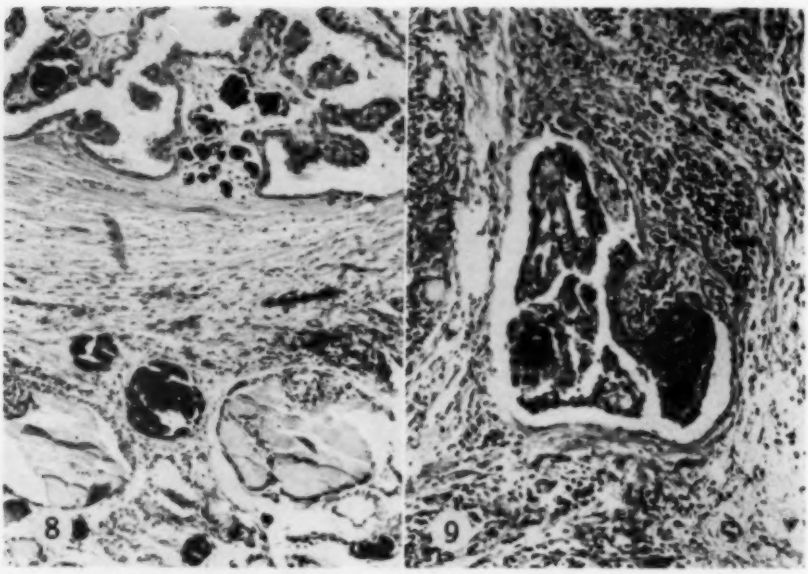


Fig. 8.—Psammoma bodies in cores of papillae and isolated in otherwise normal adjacent thyroid. Hematoxylin and eosin; × 100.

Fig. 9.—Lymphatic invasion. Tumor is focally adherent to the endothelial lining of the lymphatic space and contains a psammoma body. Hematoxylin and eosin; × 125.

170 cases (75%) and usually diffusely distributed throughout many rather than confined to one or a few tumor fields (Fig. 9). Venous invasion (Fig. 10) was found in 22 cases (10%) and was often but not invariably accompanied by lymphatic invasion (17 cases, 77%). Infiltration of tumor through capsular or pseudocapsular stroma occurred in 28% of the total cases.

Multiple microscopic foci of tumor within the gland (Fig. 11), forming discrete and separate nests, independent of the main tumor, were found in 46 cases (20%). These foci were frequently accompanied by lymphatic invasion (36 cases, 78%) and topographically were closely related to intralymphatic tumor emboli. Hence they were considered probably to be intraglandular metastases rather than multicentric primary tumors.

A radical neck dissection was performed in 62 of the cases (26%). Of these, 47 (76%) showed metastatic tumor in one or more lymph nodes.

Coexisting Lesions.—Commonest of the coexisting lesions (Table 4) was adenomatous goiter, found in 62 (28%) of the specimens. Chronic thyroiditis, nonspecific

TABLE 3.—*Type of Invasion*

	Cases	
	No.	%
Lymphatic	170	75
Venous	22	10
Capsulostromal	63	28
Intraglandular	46	20

TABLE 4.—*Coexisting Lesions*

	Cases	
	No.	%
None	122	54
Adenomatous goiter	62	28
Chronic thyroiditis	21	9
Primary hyperplasia	12	5
Separate adenoma	9	4
	226	100

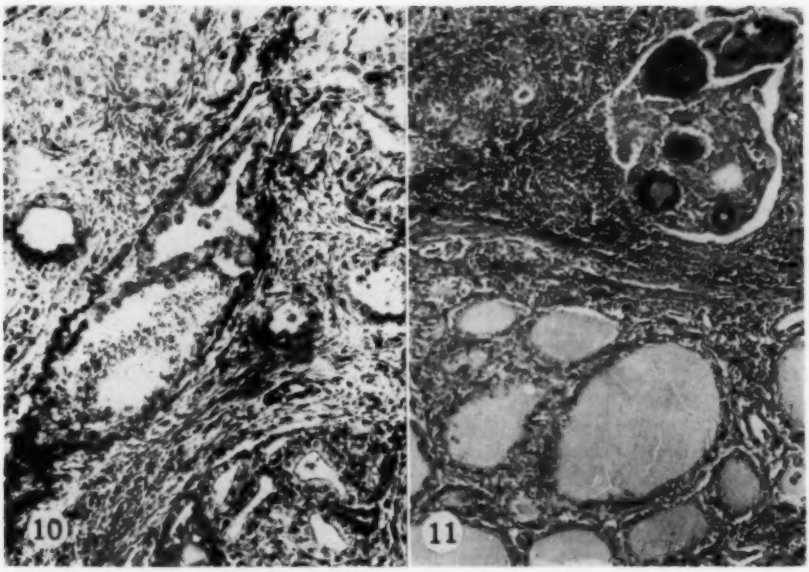


Fig. 10.—Blood vessel invasion. Verhoeff's stain; × 125.
Fig. 11.—Intraglandular metastasis. Hematoxylin and eosin; × 100

in type, was present in 21 cases (9%), and primary hyperplasia was found in another 12 (5%). Of the 12 carcinomas coexisting with primary hyperplasia, 7 (58%) were clinically unsuspected microscopic findings in glands removed solely for treatment of Graves' disease.

A separate adenoma, topographically independent of the papillary carcinoma, occurred in nine cases (4%). The criteria for the diagnosis of adenoma were as defined by Warren and Meissner.[6] In type, seven were follicular and two were Hürthle-cell. None had papillary foci, and none showed invasion.

Comment

Structure.—The great structural variability of papillary carcinomas is apparent. Variation occurs not only in cell type, to include such forms as clear cells and Hürthle cells, but also in degree of papillary formation. The decision to include in this series only those carcinomas having at least 50% papillary structure is, of course, arbitrary, for there are numerous thyroid cancers that contain papillary foci to a smaller degree. There are, in fact, all degrees of transition between pure papillary carcinoma and other types of thyroid cancer.

The incidence of squamous metaplasia in this series is higher than the 16% found by Klinck and Menk.[7] These authors have shown that squamous metaplasia in thyroid epithelium is not specific to neoplasms, since it may also be found in chronic thyroiditis and adenomatous goiter. This metaplasia did not parallel the degree of stromal inflammation but rather appeared to be a special growth characteristic of the individual tumor.

The psammoma bodies frequently present in these tumors appear to arise in the fibrous cores of the papillae and are comparable to similar structures found in papillary tumors of other tissues, e. g., ovary. Isolated psammoma bodies independent of tumor are presumptive evidence of papillary tumor somewhere in the vicinity.

Origin.—A reasonable supposition regarding pathogenesis is that papillary carcinoma is somehow related to hyperplasia.

Certainly the tumor papillae, and even the individual tumor cells, show considerable structural resemblance to the papillary hyperplastic processes found in the primary hyperplasia of Graves' disease and the secondary hyperplasia of adenomatous goiter. Also, the frequent occurrence of tumor clear-cell foci suggests pituitary TSH effect analogous to that which may be operative in hyperplasia.[8] Although papillary tumors appear to be functionally inactive, failing to form colloid in typically papillary foci or to show significant affinity for radioiodine,[9] this fact does not in itself necessarily exclude basic relationship between them and hyperplasia. The 5% incidence of coexisting primary hyperplasia is of considerable interest. This figure may be weighted, however, inasmuch as glands removed for treatment of Graves' disease may also contain small occult carcinomas. Indeed, of the 12 cases of papillary carcinoma found coexisting with primary hyperplasia, 7 (58%) were clinically unsuspected.

It is difficult to establish any distinct relationship of these tumors to pre- or coexisting adenomas. A separate adenoma was found in 9 of the 226 cases in this series, but none of these appeared more than coincidental. From studies reported elsewhere,[10] it seems unlikely that papillary carcinomas arise from preexisting adenomas.

Other coexisting lesions, namely, adenomatous goiter and chronic thyroiditis, did not occur with a frequency that could be considered more than coincidental.

There is no definite evidence that these tumors arise from remnants of the thyroglossal duct or other primitive epithelium. Thyroglossal duct epithelium may include stratified squamous and ciliated types. It is true that papillary carcinomas often undergo squamous metaplasia, but in no case were true cilia demonstrated. Furthermore, the tumors showed no predilection for localizing within the midline pyramidal lobe or isthmic regions of the gland, and although cases have been reported in the prepubertal age group, some even in infancy,[11] the great majority of these tumors are first clinically recognized in adult life.

Growth Potential.—By definition no papillary tumor was considered malignant unless invasion was found. This criterion is necessary because the relative lack of pleomorphism and the almost complete absence of mitoses in these tumors preclude estimation of malignant potential from cytology alone. Furthermore, both primary hyperplasia and adenomatous goiter may show papillary processes and thus be mistaken for cancer unless the criterion of invasion is applied.

It is not surprising that such slow-growing tumors as papillary carcinomas are often regarded and treated in a manner somewhat different from other faster-growing cancers. However, the slow growth rate of these tumors should not be confused with lack of malignant potential. Papillary carcinomas are true cancers, as is amply evidenced by their ability to invade and metastasize.[12]

Summary

The pathology of 226 surgical cases of papillary thyroid carcinoma was studied with regard to occurrence, gross appearance, microscopic variations, and growth potential.

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Microscopic Criteria of Carcinoma and Sarcoma

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Introduction

In training medical students (and dental students), as well as interns and residents, some difficulty is encountered in giving them a basis for the microscopic determination of carcinoma and sarcoma. Actually the latter causes more concern than the former. Frequently, so much emphasis has been placed upon mitotic figures that other and significant factors are either ignored, forgotten, or possibly never even learned. The importance of this concept will prevail as long as the determination of malignancy rests on this diagnostic method.

There is no intention to ignore the use of gross changes, biologic behavior, roentgenograms, and clinical data. These have been stressed in many papers, texts, and presentations. Their value is readily accepted. However, in the majority of instances final determination hinges on the cellular and organoid alterations.

Primarily, an adequate knowledge of normal histology must be presumed. Such a basis can be the only foundation for the interpretation of the abnormal. If necessary, a review is requested and made helpful by having or using a set of normals.

By reading the "Lectures on Tumor Pathology" [1] given to the Cornell students by Dr. James Ewing, it was possible to formulate the following chart utilized in carcinomas. This was also elaborated in his text on "Neoplastic Diseases" [2] and by the perusal of other books and journals.

Microscopic Criteria

1. Overgrowth
2. Varied blood supply
3. Break in basement membrane
4. Invasion of tissue, blood vessels, lymphatics alone or in any combination, and of adjacent structures and/or organs
5. Desmoplasia
6. Metastasis
7. Heterotopia
8. Loss of polarity and pseudostratification
9. Atypical cells—varied size, form, staining; hyperchromatism; mitoses (atypical, asymmetrical, polypolar); tumor giant cells

This is typed by the secretary and given to all new members in the department. Also, use is made in conversation with clinicians on certain cases. Actually, it is used as a reference at all times when slides are being studied. Again a slight digression: One assumes that the choice of tissue sections for study requires sufficient knowledge of gross neoplasms so as to select the proper abnormal regions for histologic interpretation.

The definition of terms has been well established. Some variation in semantics may be permitted. Most of the words and phrases are clear. In explaining their meanings certain latitude is permitted.

Overgrowth is considered to be a degree beyond ordinary hyperplasia. Varied blood supply is thought of as an increase in vascularization in an attempt to accompany progression of the carcinoma. A break in the basement membrane as well as local invasiveness are thought to be self-explanatory. Extension into supporting tissues by abnormal epithelial cells or glands should be readily recognized, also penetration of the walls of blood vessels and lymphatics. Presence in adjacent organs of the neoplastic cells by contiguity requires no comment—it does presume a knowledge of gross anatomy. Desmoplasia is interpreted as fibrous proliferation in an attempt to obstruct growth. Emphasis is placed on recognizing groups of abnormal cells. Metastasis and heterotopia are also defined

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in specific terms. Examples of polarity loss and os pseudostratification are shown, and then a crude drawing is made to explain them.

Because so much stress has been laid on the concept of increased mitotic activity, the varied forms are demonstrated so that in turn the student or the resident can recognize them. Symmetrical and asymmetrical mitoses are charted. Varied size and staining of the cells are also impressed upon them. Finally, the tumor giant cells seen in many carcinomas are emphasized.

When the various criteria have been taught and an attempt made to apply as many as is possible in each case, then it is feasible to indicate that all manifestations of activity are not present in each carcinoma.

With the passage of time, more and more reliance is placed on all aspects rather than on one individual characteristic. On the other hand, it is rather obvious that if invasion is readily recognized, many of the other factors are simply additive.

In December, 1942, in association with Dr. Shields Warren, a paper on "Leiomyosarcoma of the Uterus" was published in the *Annals of Surgery*.[3] As the basis for it the following changes were utilized in determining the existence of malignancy for connective tissue tumors.

Microscopic Criteria of Sarcoma

1. Increased cellularity
2. Increased mitoses
3. Pleomorphism
4. Bizarre nuclear forms
5. Hyperchromatism
6. Increased nuclear-cytoplasmic ratio
7. Loss of pattern or architecture
8. Anaplasia (dedifferentiation)
9. Multinucleation
10. Tumor giant cells
11. Invasion
12. Lack of capsule
13. Gradation from normal
14. Retained fibrous stroma
15. Vascular slits
16. Degenerative changes (hemorrhage, cysts, hyalin, necrosis)
17. Blood vessel invasion
18. Metastases

Although most of these are self-explanatory and some of them have already been delineated in the section on carcinoma, others require more comment.

The increased cellularity is well recognized by comparing with a normal segment of the tissue. Also, there is usually less intercellular substance and usually minimal collagen. Mitoses and pleomorphism have been described. Bizarre nuclear forms are very marked in sarcomas. Increased nuclear-cytoplasmic ratio is a most helpful finding and, of course, a part of the cellularity. Tumor giant cells become noticeable in association with this increased ratio as well as with the bizarre nuclear forms. Often multinucleation is an outstanding aspect. Invasion and lack of capsule are sometimes synonyms—often each is present, and occasionally, either one or the other. When retained, gradation of the normal to the abnormal is also stressed. Vascular slits are recognized much more frequently in sarcoma than in carcinoma. They consist of a thin endothelial layer with a fine stroma. Actual degenerative changes usually occur more often in those instances where the growth is so rapid as to interfere with the blood supply. Sometimes the vascular slits and the veins are invaded by tumor cells. There may even be true thrombi with complete occlusion of the lumen. These in turn contribute to the degenerative changes already mentioned. Of course the presence of true metastasis in other tissues or organs is sufficient in most instances to affirm the diagnosis.

As one might anticipate, not all sarcomas have each one of the changes mentioned in the above list. Nonetheless, the greater the number present, the more certain is one in his interpretation.

At least, reliance is not placed upon one single factor, such as is the tendency in some quarters.

There are obviously some groups of malignant neoplasms not necessarily covered

by either of these tables, namely, the gliomas and the lymphomas. Nonetheless, with minor variations and with slight changes the criteria may still easily apply. Invasion of the capsule is important in lymphomas and multinucleation, and tumor giant cells are common in the more advanced gliomas.

Degenerative changes and blood vessel alterations are frequent in the gliomas.

Hence, by the use of many of these criteria, it is possible to be more certain of the accuracy of diagnosis of malignancy.

Summary

Two tables, with some explanatory comments, have been suggested for use in the diagnosis of carcinoma and of sarcoma.

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The Production of Osteogenic Sarcomas in Rats with Radioactive Calcium

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These experiments were started in 1951 in an attempt to determine how much Ca^{45} might be administered to a rat without producing injury. A preliminary report on some of the results was presented by Barnes and McCay,[1] in 1954.

Martland and Humphries,[2] in 1929, discovered that the continuing effects of ionization radiations on bone produced osteogenic sarcomas after a considerable latent period. Their observations on humans were soon corroborated by experiments on animals [3,4] and by detailed clinical reports including the notation of carcinomas of the nasal sinuses as well as sarcomas in bone.[5]

The effects of radioactive isotopes of a number of the elements which concentrate in osseous tissue have been studied. Lisco, Finkel, and Brues [6] reported the occurrence of late osteogenic sarcomas in experimental animals following the injection of plutonium and the radioactive fission products—strontium (Sr^{89} and Sr^{90}), yttrium (Y^{91}), and cerium (Ce^{144}). The latter three are pure β-emitters with maximum energies of 1.5, 1.5, and 0.35 mev and half-lives of 55, 57, and 275 days, respectively. The localization of these elements in bone is discussed in detail by Heller in Bloom's publication of some of the experimental data from the Manhattan project.[7] Koletsky, Bonte, and Freidell,[8] in 1950, reported the production of osteogenic sarcomas in various bones and carcinomas of the nasal sinuses of rats by the administration of P^{32}. A long list of radioactive isotopes deposited in the mineral lattice and osteoid matrix of bone and in cartilage is now available in an excellent book by McLean and Urist.[9] The carcinogenic effects of radioactive calcium on the bones of mice were demonstrated by Anderson, Zander, and Kuzma, in 1956.[10] They also quoted similar effects in rats.

Ca^{45} is a pure β-emitter, maximum energy 0.254 mev and half-life about 163 days. In these experiments the Oak Ridge preparation Cat. No. Ca^{45} P-2 was used. This preparation has a sufficiently high specific activity that it could be given orally without exceeding the rats' daily calcium requirement. In vivo [11-13] and in vitro [14] experiments with the uptake of Ca^{45} have shown that calcium is promptly and quantitatively taken up by growing bone and that a sizable amount of it is rapidly converted to a relatively nonexchangeable state by crystallization and recrystallization processes.[15] A similar avidity of ossifying cartilage for Ca^{45} has been demonstrated by radioautographic in vitro techniques.[16] Because of the increasing use of Ca^{45} in tracer studies and in our own case because of our interest in long-time studies of calcium metabolism, it was thought desirable to study the dosage level at which the β-particles from Ca^{45} might injure the animal in some way.

Methods

The white rats used in these experiments were from the Cornell rat colony, which originated in

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Incidence of Osteogenic Sarcomas *

Group †	Rats, No.	Doses, No. ‡	Av. Total Dose/ Gm. of Rat, μc.	Total Dose/ Rat, μc.	Rats with 1 or More Sarcomas, No.	Rats with Metastases, No.	Mean Span of Life, Days
I	11	9	0.49	42	0	0	457
II	11	15	0.89	106	0	0	522
III	17	6	1.13	200	1	0	644
IV	13	30	2.85	360	1	1	478
V	13	29	4.75	785	12	8	314
VI	20	6	5.65	971	15	9	279

* Not included in the above is one osteoma and one rhabdomyosarcoma.
† Groups I, II, IV, and V were male rats; Groups III and VI, both male and female rats.
‡ All doses started when rat was 30 days of age.

the Yale colony some 30 years ago. The rats were placed in individual cages at weaning time and were given 10% sugar solution in drinking tubes in addition to our stock diet. At 27 days of age they were divided into groups (Table) and changed from the stock diet to one which was barely adequate in calcium. They were continued on this diet throughout the period of Ca^{45} intake and for 48 hours after the last dose of Ca^{45}, when they were returned to our stock diet. The Ca^{45} was administered by mixing it with the 10% sugar solution. The doses were started at 30 days of age in all groups.

The dosage schedule is indicated in the Table. The rats in Groups I, II, IV, and V (our first experiment) received Ca^{45} at 48-hour intervals throughout the dosage period. In Groups III and VI (our second experiment) the dosage schedule was interrupted because of the illness of one of us. These rats received the first four doses at weekly intervals and, after skipping four weeks, received the remaining two doses at weekly intervals.

Blood cell counts were taken at about monthly intervals on all rats from Groups I, II, IV, and V, starting at or before the end of the dosage period and continuing for a period of about six months.

For a period of nearly 200 days after the last dose all rats appeared to be normal. The growth curves for rats of the same sex were the same for a group on high dosage of Ca^{45} as for the group on the lowest level. No significant differences in blood cell counts between the highest level and lowest level group could be demonstrated. This might be surprising in view of the well-known effects on the blood picture produced by β-particles from P^{32} and Sr^{90}, but one may recall that the β-particles from Ca^{45} have only about one-sixth the maximum energy of the ones from P^{32} and Sr^{90}.

The first abnormalities to appear were among the rats in Groups V and VI, either a rapidly growing bone tumor or a paralysis of the hindlegs. The paralysis of the hindlegs was found later to be accompanied by the development of a tumor somewhere on the spinal column. Three obviously moribund animals were killed. The time interval between the first appearance of a tumor or paralysis of the hindlegs and death was approximately 20 days.

At death the rats were x-rayed in two different positions, were autopsied, and were either frozen or eviscerated and placed in 10% formalin. Some months later a more detailed study of the carcass was made and sections were taken from the viscera and bones which were fixed in 10% formalin. In animals with no x-ray-evident bone tumors routine sections were taken of the ends of the femur or humerus and of the vertebrae. Thin sections were decalcified by continuous rotation in 5% nitric acid, usually for 24 hours. All sections were stained with hematoxylin and eosin after paraffin imbedding. Bone tumors were stained in addition with Rinehart Abul Haj colloidal iron, periodic acid-Schiff stain (P. A. S.), alcian blue, and P. A. S.-alcian blue[17] stains for mucopolysaccharides, and with von Kossa silver nitrate stain for calcium.

Results

None of the rats of Groups I and II developed bone tumors. One rat in Group III and one in Group IV developed bone sarcomas. In Group V, 12 of the 13 rats had one or more bone tumors. In Group VI, 16 of the 20 rats had one or more bone tumors (1 was classified as osteoma, 15, as osteogenic sarcoma). Two of the four rats which showed no tumor had paralysis of the hindlegs, which was characteristic of all of those rats with a tumor on the spine.

Forty-three osteogenic sarcomas were found in 29 animals. Thirty were located in long bones (humerus, tibia, fibula, and femur); seven, in vertebrae; three, in rib

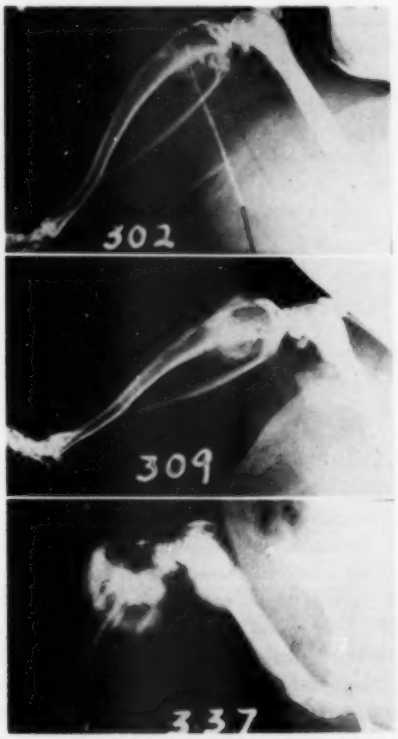


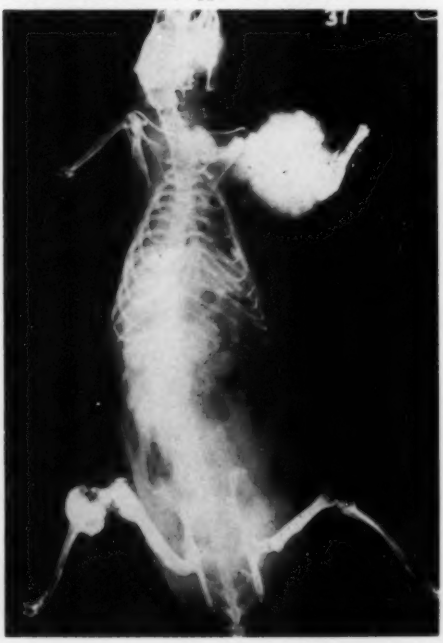
Fig. 1 (Rat 37).—X-rays showing rapid development of sarcomas at distal end of femur and proximal end of tibia at days indicated.

or sternum, and three, in the shoulder area (scapula, clavicle, or head of humerus). Tumors of long bones, when small enough for a site of origin to be discerned, usually occurred near the ends of the shaft, suggesting an origin at or near the position of the epiphyseal line during the dosage period. Figure 1 shows a series of three x-ray pictures of the hindleg of a rat from Group V. These pictures were taken at ages 302, 309, and 337 days, respectively, and show the rapid growth of two tumors, one originating a few millimeters from the proximal end of the tibia and the other, a few millimeters from the distal end of the femur. The rat was dead at age 337 days.

Several animals had multiple primary tumors; 1 had four (Fig. 2); 2 had three; 7 had two, and 19 had one. An additional animal had a spindle-celled tumor in contact with the femur which we classified as a rhabdomyosarcoma because of the strap-cell appearance of some tumor cells and the absence of osteoid. Two animals had marrow infiltrations resembling lymphoma. Some animals in the high-dosage groups showed foci of osseous proliferation with and without accompanying fibrosis (radiation osteitis). Most of these foci were composed of fairly well-differentiated osseous trabeculae appearing irregularly along preexisting spicules of bone. One nodule was composed of mature bone trabeculae and was classified as an osteoma.

The highest incidence of sarcoma appears in Group V, but the results in Groups V and VI should not be compared too precisely because of the four-week interruption during dosage of Group VI. When one compares Group V (785μc. in 29 doses) with Group IV (360μc. in 30 doses), it appears that somewhere between the two dosage levels lies the point at which enough

Fig. 2 (Rat 37).—X-rays of one rat showing four sarcomas: humerus, upper end of sternum, lower end of femur, upper end of tibia.



isotope is absorbed and deposited to produce sufficient β-emanation for consistent tumor production, not only at one but at many sites.

Gross Findings.—The tumors appeared as nodular swellings which when small and in long bones were found a few millimeters from the end of the diaphysis. When large, these surrounded the entire bone, bulging into adjacent structures. Some appeared to grow across joints into contiguous bones, making the point of origin difficult to ascertain, although two primary tumors may have fused to produce this effect. The edges of the tumors were pseudoencapsulated. Usually the outer portions of the multinodular masses were soft, fleshy, hemorrhagic, and in places necrotic, while the centers were either frankly osseous or at least gritty. Only rarely was ossification entirely absent grossly. The metastatic lesions in the lungs could usually be identified grossly. Some were granular, suggesting early calcium deposition. The internal viscera otherwise appeared normal.

Microscopic Examination.—These tumors exhibited much the same variation in pattern as is seen in human osteogenic sarcomas. All of the tumors except one showed variable amounts of osteoid or chondroid matrix. Most of the tumors were richly cellular and highly invasive at their edges but contained mature osteoid elements centrally. The latter, often appearing as well-differentiated osseous spicules, were laid down in juxtaposition to endosteum or periosteum of residual fragments of bone. Figure 3 shows such osseous trabeculae attached to one surface of a piece of cortical bone, while undifferentiated tumor cells growing along the other surface invade voluntary muscle.

In general, the tumor cells were pleomorphic, varying from polygonal to triangular to fusiform. Nuclei were large, round, or oval and actively mitotic. A few tumors were very anaplastic; yet osteoid spicules could be found in nearly all of them. In some tumors, cartilage was seen initially as the abundant intercellular component. In the hematoxlyin-and-eosin-stained sections foci of basophilia were seen frequently in this cartilagenous matrix (Fig. 4). In the more central and presumably older parts of the tumor these fused with laminated spicules and were interpreted as osteoid. These intracartilaginous strands took on a deep magenta color with periodic acid-Schiff stain and various depths of blue with alcian blue-periodic acid-Schiff and colloidal-iron stain (Abul-Haj), suggesting the early appearance of an aldehyde-producing poly-

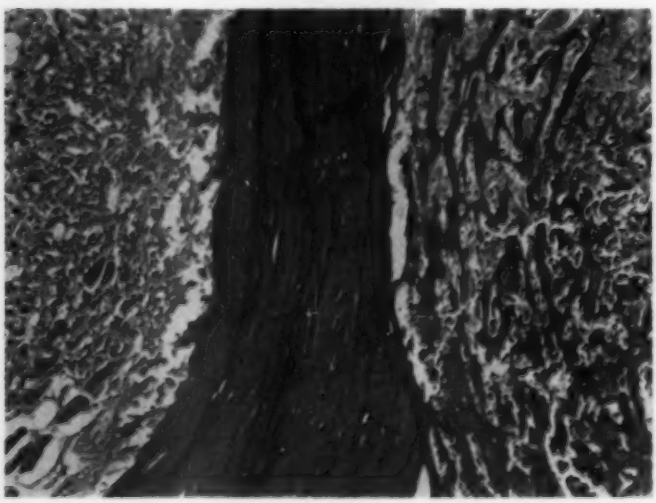


Fig. 3.—Spicule of mature bone with osteoid trabeculae attached to one surface and undifferentiated tumor cells growing against the other. The latter invade voluntary muscle. Hematoxylin and eosin stain.

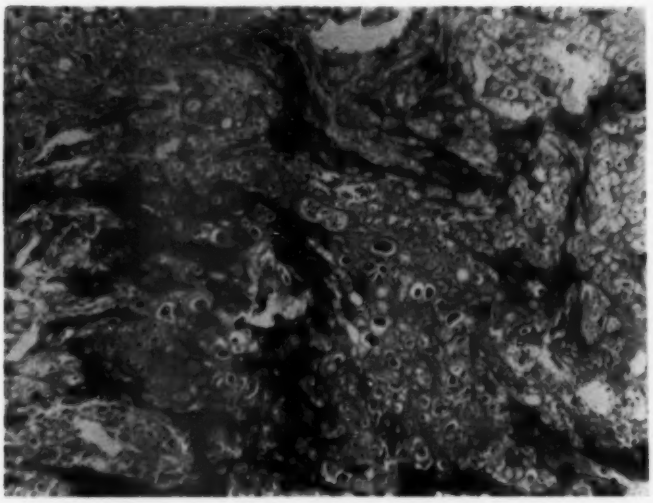


Fig. 4.—Basophilic foci developing in cartilaginous matrix. Elsewhere these fuse with frank osteoid strands seen in Figure 3. Hematoxylin and eosin stain.

saccharide in the ground substance as well as cartilage-like acid mucopolysaccharide. Rare tumors remained predominantly cartilagenous, and in these only a few questionable osteoid spicules were noted (Fig. 5).

In many other tumors, clear cells resembling cartilage were not seen in the ground substance and strands of periodic acid-Schiff-positive interstitial substance appeared to develop directly adjacent to tumor cells. These also exhibited some metachromasia, suggesting the presence of acid mucopolysaccharides. They fused in places with laminated osteoid spicules and thence with strands of immature bone, as seen in Figure 3. Thus the genesis of bone in the entire group may take place in two ways: either directly from ground substance or indirectly by intracartilaginous ossification as seen in the embryonic epiphyses. Foci of calcification appeared early in both the osteoid and osteochondroid foci, as seen in the von Kossa stains.

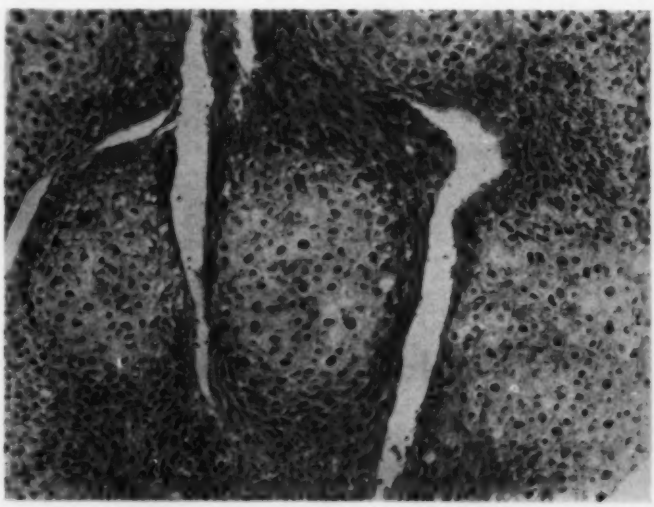


Fig. 5.—Neoplastic cartilage resembling foci of embryonic chondrification. Hematoxylin and eosin stain.

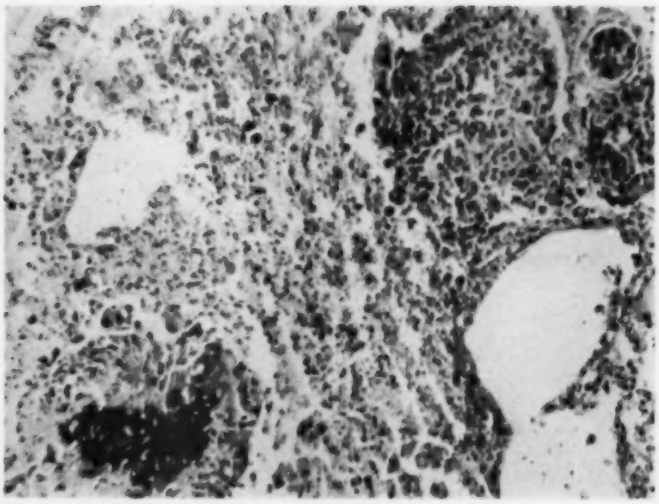


Fig. 6. — Two lung metastases. In the upper right a small entirely cellular tumor embolus and in the lower left an older osteochondroid metastasis with central calcification. Von Kossa stain.

Metastatic foci from these osteogenic sarcomas resembled the primary tumors closely. While early metastases were entirely cellular, those of any size contained clearly discernible osteoid or osteochondroid strands. Black calcium granules were seen with von Kossa stain in early metastases, indicating the rather prompt creation of a matrix suitable for calcification (Fig. 6).

Comment

Twelve of the thirteen rats in Group V developed osteogenic sarcomas in one or more bones. Thus, Ca^{45} given to young growing rats in sufficient dosage over a two-month period appears to be a potent sarcogenic isotope. Involvement of the upper end of the humerus, the lower end of the femur, and the upper end of the tibia as frequent primary tumor sites is consistent with the fact that these parts of the skeleton grow rapidly during this phase of life and take up Ca^{45} most avidly. The presence of a well-defined cartilaginous matrix in a few of the tumors and of chondroid components in others suggests that Ca^{45} was taken up by calcifying cartilage. This might be expected, since these rats received the isotope during a period of active calcification of cartilage and since the affinity of Ca^{45} for cartilage has been demonstrated.[16] Koletsky [8] reported seeing cartilage in some of his P^{32} rat tumors, and Anderson et al. mention it as occurring in mouse tumors arising from Ca^{45}.

As might be anticipated, the rats with multiple osteogenic sarcomas developed metastases more frequently than those with single tumors. All 10 animals with more than one primary tumor had metastases, while only 9 of 19 with single primary tumors showed this change. The lungs were involved in all animals showing metastases. Other sites with secondary tumors were lymph nodes, three; heart, one; pericardium, one; adrenal, one, and kidney, one.

Summary

Ca^{45} is a potent agent for the production of osteogenic sarcomas in the skeleton of the rat, provided it is given in sufficient repeated doses during the period of rapid growth of the skeleton.

In Group V the average interval from the last dose of Ca^{45} until the appearance of a tumor or paralysis of the hind legs was 207 days. The average time from the appearance of tumor or paralysis until death was 21 days.

In Group VI the average interval from the last dose until appearance of a tumor

or paralysis was 155 days, and the average interval from the first appearance of a tumor or paralysis until death was 26 days.

Ten of the twenty-nine tumor-bearing rats had multiple primary osteogenic sarcomas. Metastasis occurred in all of these. Nine of the remaining nineteen rats with single osteogenic sarcomas had metastases.

The considerable amount of neoplastic cartilage in some of these tumors suggests that Ca^{45} is deposited in calcifying cartilage as well as in bone.

Total doses of less than 1μc. per gram of rat are not likely to produce osteogenic sarcoma.

Ca^{45}, as used here, produced no effect on the blood cell counts of the rats during the ensuing six months.

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A Complex of Glioblastoma and Spindle-Cell Sarcoma with Pulmonary Metastasis

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The association in the brain of malignant glioma and primary sarcoma of nonteratomatous nature has long been known although few cases have been recorded. This may have been due in part to the pleomorphic appearance of glioblastomas, which lends itself to misinterpretation. However, since the glial origin of these tumors has been unequivocally established and the morphology and staining reactions of malignant connective tissue tumors have become well known, it is possible to distinguish with reasonable certainty between the two groups of neoplasms, even when present in a single tumor. This is particularly valid in the central nervous system, where the only mesothelial elements present are the meninges, blood vessels, and microglia.

Feigin and his colleagues [16,17] have reported 16 cases of coexistent glioblastoma and sarcoma. Of these, 6 had been recognized in a series of 433 consecutive brain tumors of which 313 were gliomas. The other 10 cases were from other sources (1 of these is the subject of this report). The tumors were all located in the cortex and subcortical white matter and in some instances involved the leptomeninges. All the tumors had the gross appearance of glioblastoma, and in some instances there were also firm white foci which proved to be predominantly sarcomatous. Histologically there was an admixture of two malignant tumors, a glioblastoma and a spindle-cell sarcoma. Rubinstein [32] described five cases, of which three were meningeal sarcomas associated with contiguous malignant gliomas. There are also a number of isolated case reports of similar tumors.[3,9,18-20,22,23,27,30,33] The nature and origin of these complex tumors has aroused considerable speculation. Feigin [16,17] and, in isolated instances, Bailey [3] and Rubinstein [32] believe that the glioma antedated the sarcoma and that the latter arose in hyperplastic blood vessels. Vascular hyperplasia occurs in varying degrees in many cerebral neoplasms and is especially marked and characteristic in glioblastoma.[13,15,16,21,37,39] Three varieties of vascular hyperplasia have been described (adventitial, endothelial, and glomerular). There is, however, no specific correlation between these morphologic types and the various intracranial neoplasms.[16] Others [9,22,33] (including Rubinstein [32] in two cases) suggested that the sarcoma, presumably of meningeal origin, came first and provoked a neoplastic reaction in glial tissue. Another group of observers [19,27,30] considered that the two malignant components were independent and of different origin. They would be, in effect, examples of multiple primary malignancies. And finally, one case was reported [20] in which the possibility of a glioma giving rise to a sarcoma was suggested.

Whereas intracranial metastases of gliomas are fairly common,[6] distant metastases of these tumors and of all primary brain tumors are exceedingly rare.[41] This is usually attributed to the absence of lymphatic channels in the brain, the infrequency of venous tumor invasion, and the possibility that glial tissue may not grow when transported into other tissues. That malignant gliomas can grow in distant sites is clearly demonstrated by a case of Wolf, Cowen,

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and Stewart [44] in which a glioblastoma metastasized to the pleura and diaphragm through a ventriculopleural tube. Furthermore, Zimmerman [46] has shown that experimentally induced gliomas in mice continue to grow upon transplantation. In almost all cases of extraneural metastases a craniotomy was performed, and this is usually considered to be the inciting cause of tumor transplantation. Among the documented reports the commonest are meningiomas or meningiosarcomas, of which there are 12 cases.[1,10,11,25,34,42] In the glioma group there are six glioblastomas,[5,10,12,26,31,45] two medulloblastomas,[29,35] and one oligodendroglioma.[24] In the miscellaneous group there is reported a hemangioblastoma [1] and two cases of pinealoma.[38] Dickson [14] has seen an example of glioblastoma and sarcoma metastasizing to the lung.

The case about to be reported is that of a recurrent glioblastoma multiforme in which a sarcomatous element was present. At autopsy a pulmonary metastasis was seen. This case is being recorded not only because of the great rarity of the anatomical findings but also because it is felt that description of the metastatic lesion may be of importance in understanding the histogenesis of these tumors.

Report of Case

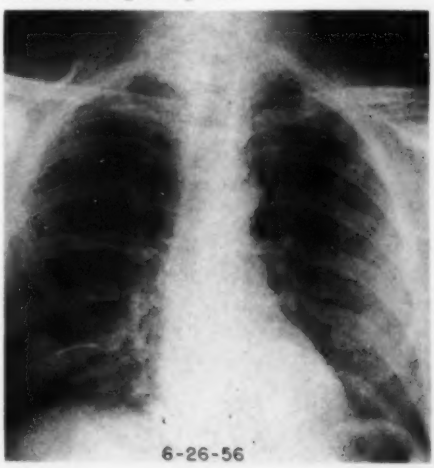
A 44-year-old right-handed heating engineer was admitted on Oct. 21, 1955, to the Veterans' Administration Hospital, Bronx, N. Y. There was a history of pain in the right shoulder and forearm for two months and severe bifrontal headaches for two weeks. Positive findings on clinical examination included bilateral nystagmus and papilledema, left homonymous hemianopsia with anisocoria (left greater than right), and left facial weakness. There were no reflex nor sensory changes. Spinal fluid showed pleocytosis and increased protein. The electroencephalogram and a right common carotid arteriogram showed evidence of a space-occupying lesion in the right temporal lobe. On Oct. 27, 1955, a craniotomy was performed by Dr. E. G. Krueger and a well-defined, apparently encapsulated, 5 cm. subcortical tumor was removed from the right supramarginal gyrus by finger dissection. The frozen-section diagnosis was "glioblastoma multiforme," and the final paraffin-section diagnosis was "glioblastoma with sarcomatous changes." After a period of relative well-being which lasted about seven months, the headaches which had been intermittent became increasingly severe and more frequent. Findings at this time were bilateral papilledema with diminished visual acuity, left homonymous hemianopsia, and a left spastic hemiparesis. On April 3, 1956, five months after the operation, roentgen examination of the chest showed a round sharply delineated 2 cm. density at the right lung base which was considered to be a metastatic lesion. All chest roentgenograms up to this time were negative. Another smaller density in the same area was suggestive of a second metastatic lesion. On subsequent roentgen examination (Fig. 1) the larger density was found to be increased in size. Roentgen examination of the skull showed recurrence of the brain tumor. Radiation therapy was started on June 26, 1956. The patient developed Cheyne-Stokes respirations, became semicomatose, and died on July 2, 1956, approximately one year after the onset of symptoms.

Gross Findings

Description of Surgical Specimen.—The surgical specimen consisted of several irregular lobulated fragments which measured approximately 9×6×5 cm. in aggregate. The largest fragment measured 9×4×4 cm. and was made up of soft multicolored tissue which appeared to be enclosed within a firm white capsule. Other fragments were smaller, white to tan, and very firm. Several pieces of white and gray matter were included in the specimen.

Autopsy Findings.—The defect in the dura at the operative site was intact and free of tumor.

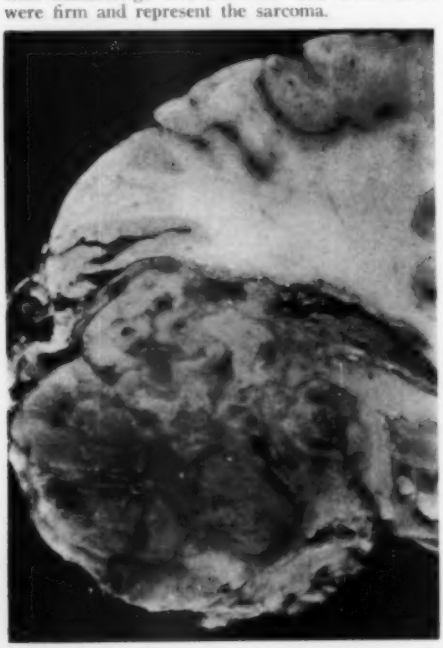
Fig. 1.—There is a rounded circumscribed density in the lower right lung field.



The brain weighed 1900 gm. The cerebral hemispheres were asymmetrical, owing to a tumor which protruded on the lateral surface of the right cerebral hemisphere over an area measuring approximately 11 cm. anteroposteriorly and 9 cm. dorsoventrally. The gyri in this region had been destroyed due to compression by the tumor. The affected gyri were the posterior half of the right superior, middle, and inferior temporal gyri; the lower extremity of the right posterior central gyrus; the major portion of the right supramarginal gyrus; the angular gyrus, and the middle inferior temporal gyri. Beyond the zone of destruction the gyri of the right cerebral cortex were broadened, and the corresponding sulci were narrowed. There was herniation of the right cingulate gyrus beneath the falx, with corresponding indentation of the left cingulate gyrus. There was marked herniation of the right uncus through the incisura of the tentorium ,or about 1.5 cm. The hypothalamic structures and the midbrain were displaced toward the left. The lateral surface of the left cerebral hemisphere showed considerable flattening of its gyri. The cerebellum and lower portion of the brain stem other than the midbrain were not remarkable. Section of the cerebrum revealed the great extent of the tumor. Its anteroposterior axis measured about 10 cm. The greatest cross sectional dimensions were 8×7 cm. There was infiltration of most of the right temporal lobe, the ventral portion of the right parietal lobe, and the anterior and lateral portion of the white matter of the right occipital lobe. The tumor extended ventrally into the convolutional white matter of the fusiform and lingual gyri and the right hippocampal gyrus. Midline cerebral structures were displaced to the left and distorted. The right lateral ventricle was partially collapsed. The tumor was firm and in some areas was very hard. It contained variegated discolored areas, yellow-green and orange, and was speckled in places by small fresh hemorrhages (Fig. 2). The hard areas were glassy and white or gray-white in color. The tumor was sharply demarcated. There was swelling and slight yellow discoloration of the central white matter beyond the margin of the tumor. Sections of cerebellum and brain stem were not remarkable.

Both lungs showed small focal areas of bronchopneumonia. In the peripheral portion of the right lower lobe, there was a single mass which measured 4 cm. in diameter. It was circumscribed and sharply demarcated from the surrounding lung parenchyma (Fig. 6A). On section, it was made up of firm discolored tissue which resembled the tumor of the cerebrum. The remainder of the autopsy was essentially negative.

Fig. 2.—Coronal section of brain, showing tumor with hemorrhage and necrosis. The white areas were firm and represent the sarcoma.



Microscopic Findings

Surgical Specimen.—The tumor was very cellular and was made up of two strikingly different types of tissue. The first consisted of spindle or fusiform cells arranged in fascicles and interlacing bundles in a fairly uniform fashion. The second was markedly pleomorphic, ranging from small hyperchromatic cells approximately the size of lymphocytes to enormous multinucleated giant cells. It occurred in clusters and sheets and showed considerable disarray and haphazard infiltration.

The nuclei of the spindle cells were elongated or oval and slender, with a fairly even distribution of chromatin. They showed moderate variation in size and shape; the larger and more irregular forms were hyperchromatic, with clumping of chromatin. Mitotic figures were present but infrequent. The cytoplasm was eosinophilic and fibrillary, with indistinct outlines. Coarse eosinophilic fibers were present between the cells and appeared to merge with the cytoplasm. The fibers gave the staining reaction of collagen (brown-red with Mallory's phospho-

tungstic acid-hematoxylin and blue with azocarmine [28]). Wilder's silver reticulum stain [28] showed a dense network of argyrophilic fibers among the cells and parallel to them. This tissue was considered to be a spindle-cell sarcoma (Figs. 5A and 5C).

The pleomorphic tissue contained a wide variety of cells. There were small hyperchromatic glial cells, often occurring in clusters. Many of the cells were much larger, polygonal, or spheroidal, with vesicular lobulated nuclei and prominent nucleoli. Present in all fields were numerous bizarre giant cells, multinucleated and often syncytial in appearance (Figs. 3 and 5B). Mitotic figures were moderately frequent. These cells were embedded in a loose, fine, fibrillary matrix which did not give the staining reactions for collagen. Some of the fibers gave the staining reactions of glial fibers (red with azocarmine and blue with Mallory's phosphotungstic acid-hematoxylin). There were no reticulum fibers with Wilder's silver reticulum stain (Fig. 5C). Areas of necrosis were numerous and were lined by palisades of small, oval, elongated, hyperchromatic cells. This tissue infiltrated the adjacent brain and was considered to be typical glioblastoma multiforme.

While there was generally close intermingling of the sarcoma and of the glioblastoma, considerable variation was seen throughout the tumor. Thus, some areas were composed almost wholly of glioblastoma, and here the occasional fascicles of sarcoma were sharply demarcated from the former (Fig. 3). In other areas, the sarcoma formed small tight whirlpools in the center of which, almost invariably, a few bizarre cells were present (Figs. 5A and 5B). In these areas, the sarcoma and the glioblastomas occasionally seemed to merge imperceptibly. However, the special stains described above provided a sharp differentiation, since the tinctorial stains for collagen and the argyrophil fibers of the reticulum stain (Fig. 5C) were never present in the glioblastoma foci, however minute.

Autopsy.—The tumor in the brain was similar to the surgical specimen. Zones of necrosis were more numerous. There were no changes which could be attributed to radiotherapy. The glioblastoma infiltrated neural tissue. There was a narrow irregular zone of intercommunicating capillaries, usually occurring in small clusters. Bizarre elongated sarcoma cells could be seen arising from the periphery of the tortuous capillaries (Fig. 8). Here, mitotic figures were numerous. The sarcoma cells were haphazardly intermingled with multinucleated giant cells and small hyperchromatic glial elements. The leptomeninges were invaded by tumor and showed orderly fibrous thickening where it occurred.

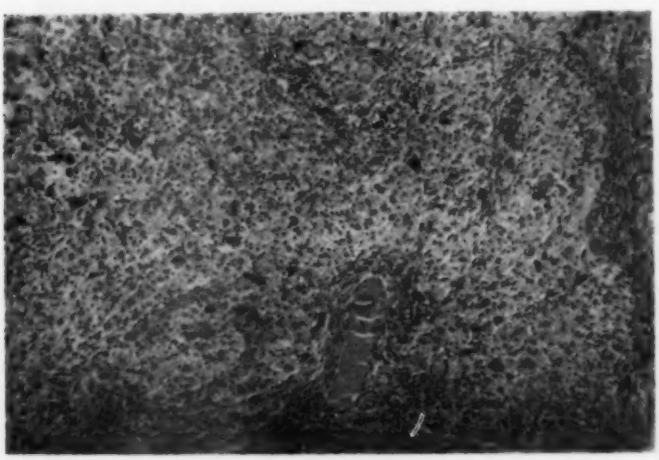


Fig. 3.—Brain tumor (surgical specimen). Predominant pleomorphic glioblastoma, with strands of sarcoma at one edge and around a blood vessel. Hematoxylin and eosin; reduced about 25% from mag. × 90.

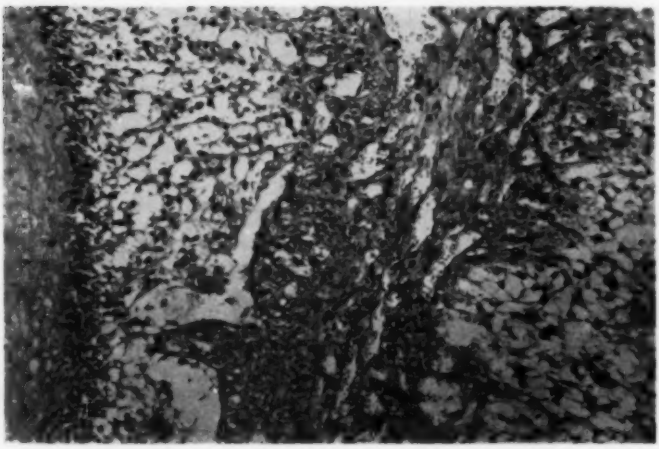


Fig. 4.—Brain tumor (autopsy). Illustrates adventitial vascular hyperplasia. Included is the edge of a zone of necrosis lined by a palisade of small hyperchromatic cells. Hematoxylin and eosin; reduced about 25% from mag. ×90.

The blood vessels in the glioblastoma area showed vascular hyperplasia of the adventitial variety.[17,21,27,39] Plump, elongated, fairly orderly cells were seen arising from the adventitia of the vessels and formed a cuff. Within the latter, small budding capillaries, often intercommunicating, were distinguished (Fig. 4). This adventitial type of vascular hyperplasia was present not only within the tumor but also in the adjacent neural tissue. Within the tumor, areas of adventitial vascular hyperplasia merged with the sarcoma. While some of the vessels contained mural thrombi, none were completely occluded by thrombosis.

Within the tumor of the lung there were large zones of necrosis lined by hyperchromatic elongated cells arranged in palisades. Both types of malignant neoplasm were present. There was generally a greater admixture of the two elements (Figs. 9A-9C). The sarcoma predominated. It was arranged in interlacing bundles (Figs. 10A and 10B) and fascicles which gave the staining reactions for collagen (blue with azocarmine, red-brown with Mallory's phosphotungstic acid-hematoxylin). The whorled appearance of the fascicles, while present, was not extensive or as striking as in the brain. There were seen reticulum fibers with Wilder's silver stain. Numerous capillaries, which often intercommunicated, were seen and were clearly delineated with the reticulum stain. In the interstices of the sarcomatous fascicles there were small islands of pleomorphic cells among which the multinucleated giant cells were prominent and were almost always present (Fig. 9A).

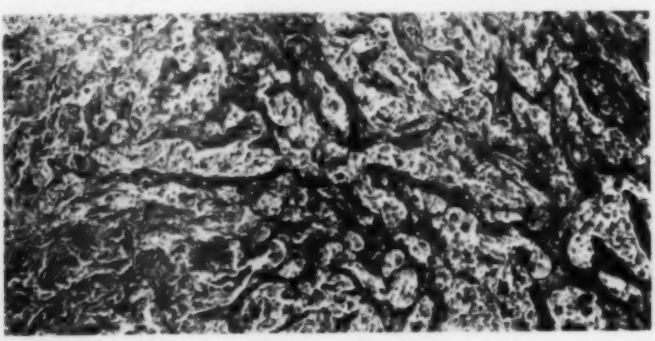


Fig. 5A.—Brain tumor (surgical specimen). Interlacing bands of sarcoma and islands of glioblastoma. The sarcoma gives the staining reactions of collagen (blue in this instance). Azocarmine; reduced about 30% from mag. ×90.

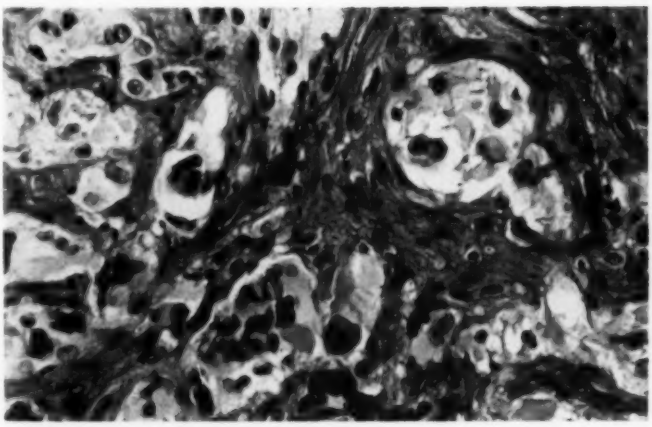


Fig. 5B. — Detail of Figure 5A. The interlacing bands of sarcoma show moderate anaplasia. The fibers are thick and coarse. The islands of pleomorphic glioblastoma are sharply demarcated from the surrounding sarcoma. Hematoxylin and eosin; reduced about 30% from mag. × 350.

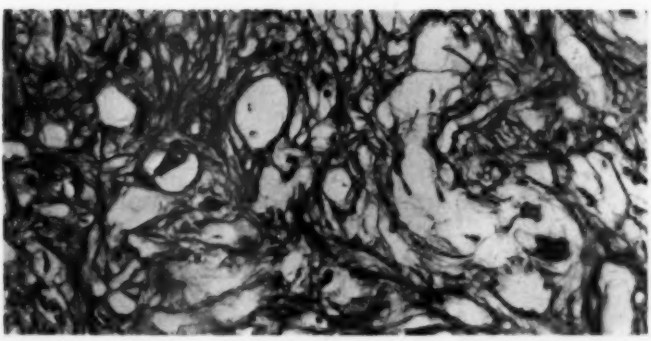


Fig. 5C.—Area similar to 5B. The reticulum fibers are abundant in the sarcomatous areas and absent in the islands of glioblastoma. Wilder's reticulum stain; reduced about 30% from mag. × 350.

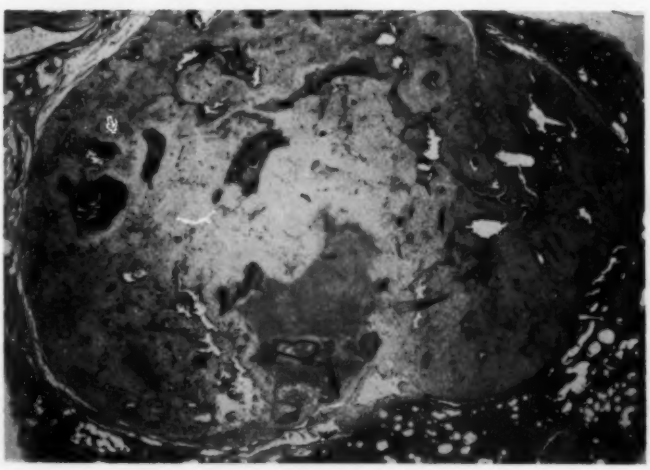


Fig. 6A. — Geographic view of lung tumor. There are large areas of necrosis and of hemorrhage. At the periphery of the lesion there is a blood vessel invaded by tumor (arrow). Hematoxylin and eosin; reduced about 30% from mag. × 5.

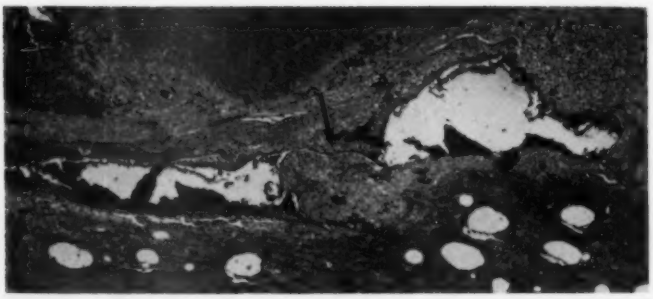


Fig. 6B. — Detail of Figure 6A. Blood vessel at boundary of lung parenchyma invaded by tumor. Hematoxylin and eosin; reduced about 30% from mag. × 20.

These islands did not contain reticulum fibers and did not give the staining reactions of collagen. Only rarely did the fine fibers give the tinctorial staining reaction of glial tissue (blue with phosphotungstic acid-hematoxylin and red with azocarmine).

The tumor was well vascularized. In places the sarcoma appeared to proliferate from the adventitia of vascular channels without invading the media and with little or no endothelial reaction (Fig. 7A-7D). In others, the sarcoma merely abutted on the

Fig. 6C.—Detail of Figure 6B. Tumor infiltrating wall of blood vessel and proliferating in the lumen. Tumor shows spindle cellular appearance of the sarcoma and the pleomorphic giant cells of the glioblastoma. Hematoxylin and eosin; × 100.

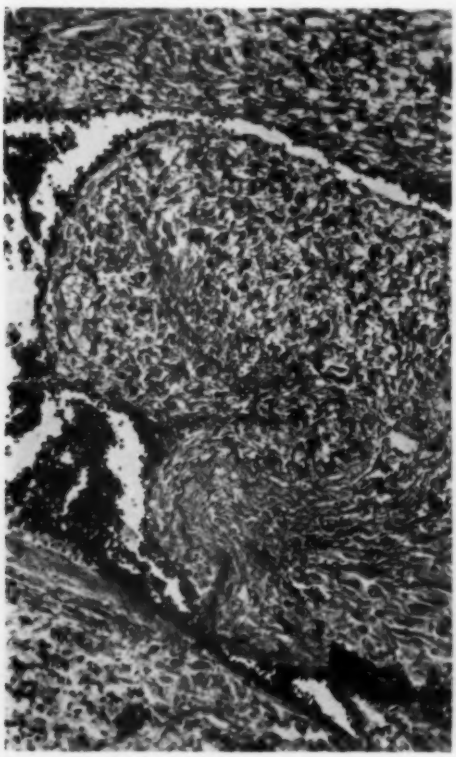
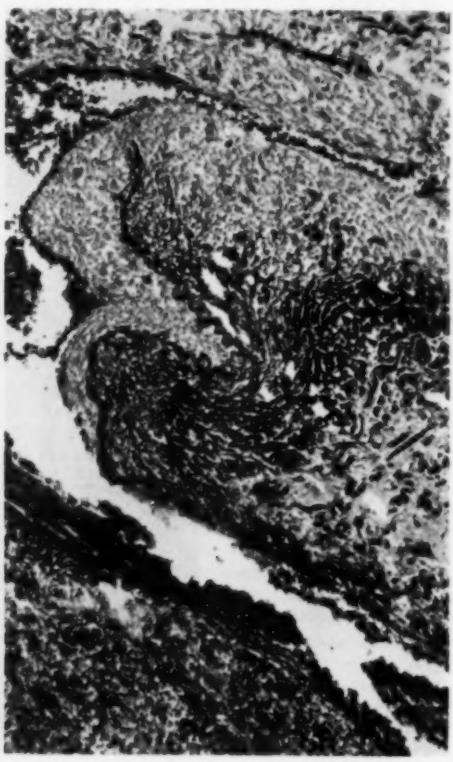


Fig. 6D.—Same field as in Figure 6C. This illustrates the disruption and destruction of the blood vessel wall and elastic layers. Verhoeff's elastic stain; × 90.



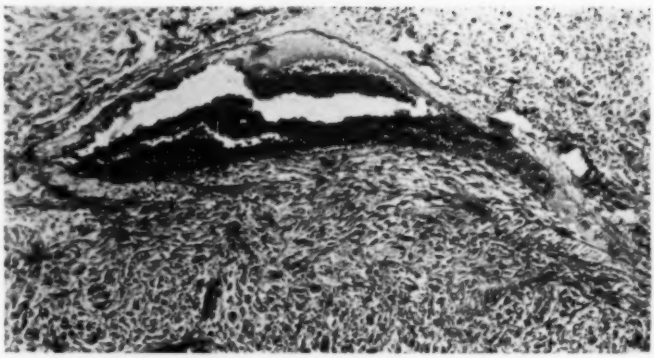


Fig. 7A.—Blood vessel in lung tumor. This shows sarcomatous proliferation contiguous with the adventitia. Small islands of glioblastoma are embedded in the sarcoma. Hematoxylin and eosin; reduced about 25% from mag. ×90.

adventitia. At the boundary between tumor and lung tissue a large vein was partly invaded by the neoplasm (Figs. 6A and 6B). Here there was infiltration of the adventitia, of the muscular coats with destruction of elastic fibers, and of the endothelium, with intraluminal proliferation (Figs. 6C and 6D). The invading tissue was principally sarcoma but with gliomatous elements present.

In the interstices between tumor cells there were several small deposits of anthracotic pigment. No residual pulmonary tissue could be seen in the tumor with hematoxylin and eosin or with Verhoeff's elastic stains. The blood vessels in the lung near the lesion showed mild thickening of the media. This change was also found elsewhere in the lung at some distance from the tumor in zones of bronchopneumonia and in a small subpleural fibrous scar. None of the varieties of vascular hyperplasia usually associated with glioblastoma were present in the lung outside of the tumor.

Comment

The lesions in the brain and in the lung were similar, and each consisted of two neoplasms which were closely intermingled. One was a typical glioblastoma multiforme, and the other, a spindle-cell sarcoma or fibrosarcoma. The staining of the sarcoma with Mallory's phosphotungstic acid-hematoxylin, azocarmine, and Wilder's silver stain clearly indicated collagenous connective tissue. In the zones of glioblastoma, collagen and reticulum fibers were absent. The rarity in these areas of fibers which give the staining reactions of glial fibers may be attributed to the anaplasia of the neoplasm. There was striking morphologic similarity between the primary brain tumor and the metastasis.

In any presumed instance of pulmonary metastasis from a neoplasm of the brain,

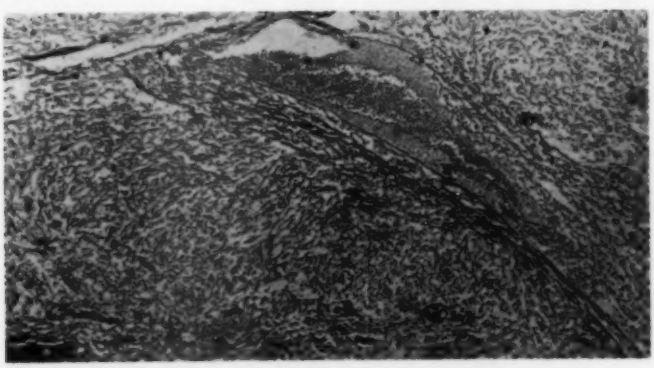


Fig. 7B.—Same field as in Figure 7A. The sarcomatous growth is adventitial without involvement of the vessel media or intima. Wilder's reticulum stain; reduced about 25% from mag. ×90.

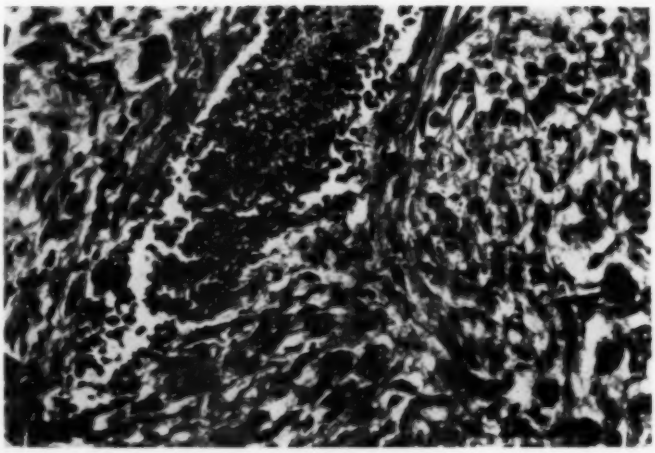


Fig. 7C.—Blood vessel in lung tumor. Proliferation of sarcoma from periphery of vessel. Immediately beyond the zone of adventitial proliferation there are interspersed small clusters of glioblastoma. Hematoxylin and eosin; reduced about 25% from mag. × 350.

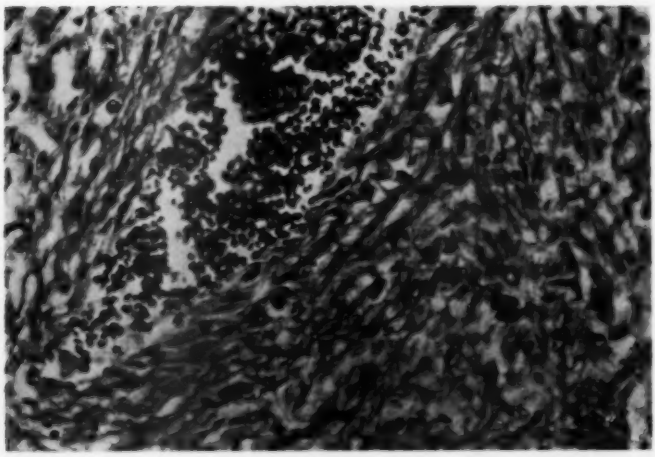


Fig. 7D.—Same field as in Figure 7C. This illustrates the adventitial growth of the sarcoma. Reticulum fibers are absent in the small foci of glioblastoma. Wilder's reticulum stain; reduced about 25% from mag. × 350.

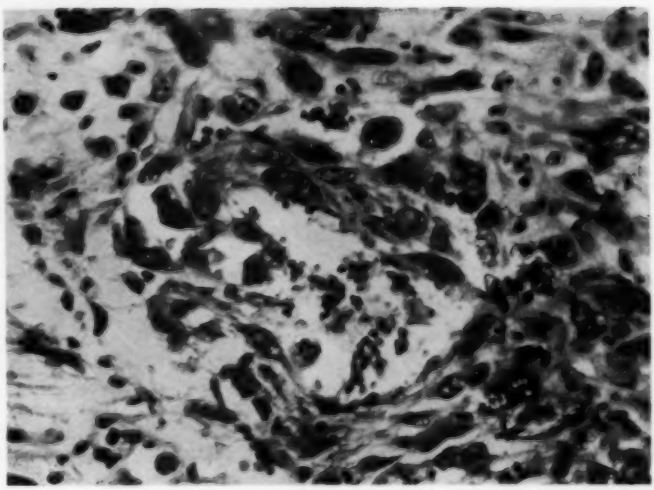


Fig. 8.—Edge of brain tumor (autopsy). Anaplastic spindle cellular proliferation from the periphery of a capillary. There is intermingling of giant cells and glial cells around the capillary. Two mitotic figures are present. Hematoxylin and eosin; reduced 20% from mag. × 350.

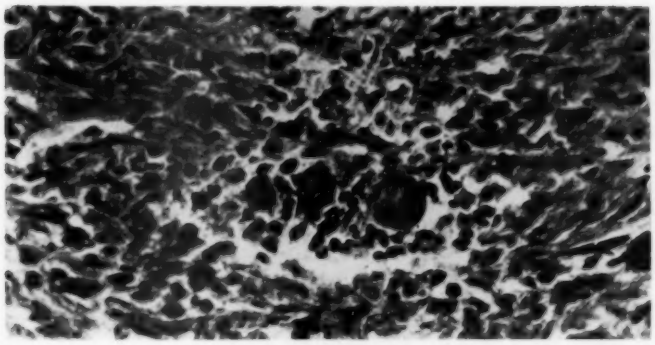


Fig. 9A.—Lung tumor. Island of glioblastoma surrounded by sarcoma. Phosphotungstic acid-hematoxylin; reduced about 30% from mag. × 350.

the possibility of a primary tumor of the lung should be ruled out. In this case there is both histological and roentgenographic confirmation of a primary brain tumor on the one hand and of pulmonary metastasis on the other. On histological examination one of the components of the brain tumor is an unequivocal example of glioblastoma multiforme, and a similar neoplasm was found in the lesion in the lung. No other malignant tumors were found at autopsy. The roentgenogram of the lung showed a rounded sharply circumscribed density in the center of the right lower lobe (Fig. 1). It was characteristic of a metastasis and not of a primary tumor. No other malignant tumors were found. Both in the brain and in the lung tumor there was marked vascular hyperplasia of the adventitial variety. While in some areas the cells proliferating from the vessels were relatively few and orderly, in others they were numerous and closely packed and showed moderate anaplasia. These cells were continuous with infiltrating sarcoma (Fig. 7A-7D). It seemed reasonable to conclude that the sarcoma in this case arose in the adventitial vascular hyperplasia, as suggested by Wolf [43] and Feigin.[16,17] The leptomeningeal surface appeared to be invaded by tumor, and in this case it is not considered to be the likely primary site of the sarcoma. Whatever doubts may have existed as to the malignant nature of the sarcomatous elements were dispelled by the rapid growth of the metastasis. There was evidence that the tumor in the lung grew by destroying lung tissue and not by expansion with compression of the lung. There were anthracotic deposits scattered throughout the tumor which were similar in distribution to the anthracotic deposits present in the uninvolved lung parenchyma. The periphery of the tumor was not limited by a capsule or by com-

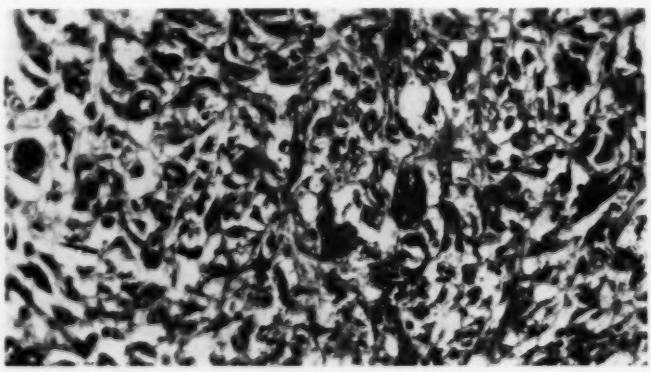


Fig. 9B.—Lung tumor. Haphazard intermingling of glioblastoma and sarcoma. Hematoxylin and eosin; reduced about 30% from mag. × 350.

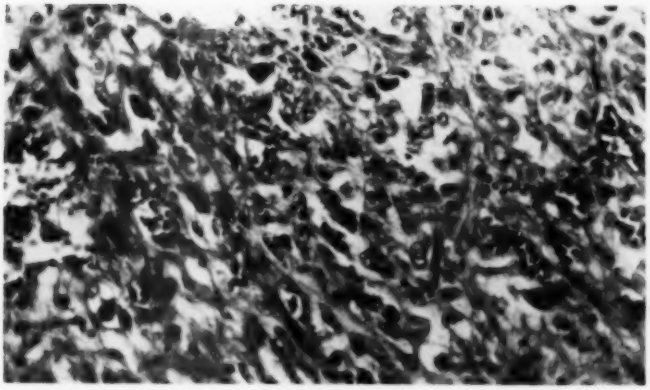


Fig. 9C.—Lung tumor. Small islands of glioblastoma encompassed within fascicles of sarcoma. Fibers of the sarcoma are coarse and thick and give the staining reactions of collagen (brown-red in this instance). Phosphotungstic acid-hematoxyin; reduced about 30% from mag. ×350.

pressed fibrous tissue. Instead there was infiltration of lung tissue, and in one peripheral area a large blood vessel was invaded and partly destroyed (Figs. 6A-6D). In all instances, both the glioblastoma and the sarcoma were present.

The presence of vascular hyperplasia in the lung tumor and its absence in the lung parenchyma outside the tumor in this case suggests that it is an intrinsic characteristic of the neoplasm and not related to the host tissue. It is possible that the factor which is responsible for the vascular hyperplasia in the primary site accompanies the tumor in its metastases and operates independently of the host tissue. Most observations of metastatic brain tumors do not mention this feature. Davis [12] states that in a case of metastatic glioblastoma multiforme vascular hyperplasia was present in the brain tumor but not in the metastatic sites. Feigin [16] also refers to the absence of vascular hyperplasia in the extraneural metastases of primary brain tumors.

In considering this complex of glioblastoma and sarcoma the following possibilities present themselves: 1. The sarcoma arose from the glioblastoma by a process of metaplasia. 2. This is a fortuitous association of two primary malignant tumors growing independently and metastasizing. 3. Both tumors are independent, but the sarcoma is derived from the vascular hyperplasia by a continuous process.

Bertrand and Medakowitch [4] tried to demonstrate in gliomas metaplasia of glial elements and suggested that the latter could give rise to fibrosarcomatous tissue. This concept has not been generally accepted. Bailey [3] states that he has never seen signs of such metaplasia in a considerable number of brain tumors. Zimmerman [46] has demonstrated that gliomas experimentally induced in animals are multipotential, one tumor

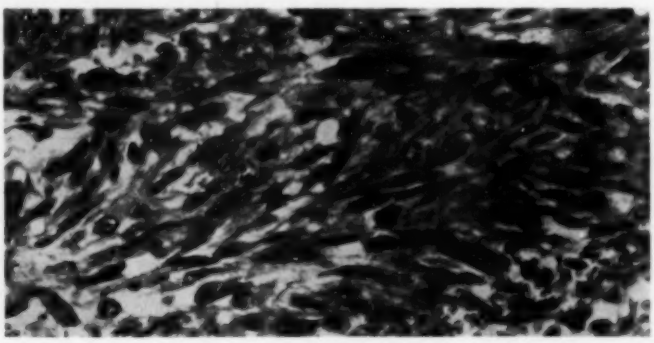


Fig. 10A.—Lung tumor. Fascicle of sarcoma, showing mild anaplasia. Hematoxylin and eosin; reduced 20% from mag. ×350.

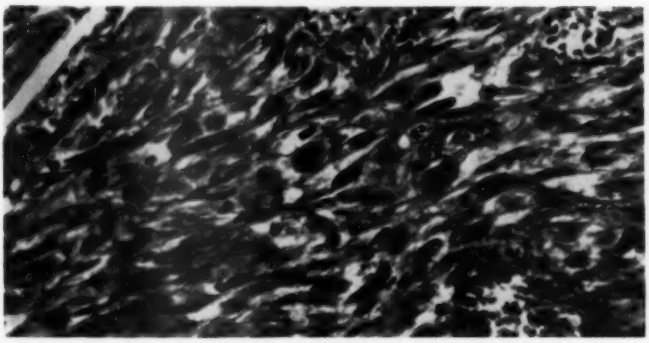


Fig. 10B.—Lung tumor. Sarcoma, illustrating moderate anaplasia. A mitotic figure is present. Hematoxylin and eosin; reduced 20% from mag. ×350.

giving rise to several types upon transplantation. A mouse glioma with the appearance of ependymoma produced an amorphous mass of tumor cells in the allantoic membrane of the chick egg but reverted to its original type when retransplanted to the mouse. However, the transformations were from one type of glioma into another and not into connective tissue tumors.

This case has many of the features of multiple primary tumors as outlined by Warren and Gates.[40] Each of the tumors was distinct; each presented a definite picture of malignancy, and there is no likelihood of one being a metastasis of the other. The close intermingling of the two neoplasms at one site might be considered to be an example of a collision tumor. But the identity of the histologic appearance in both sites renders this possibility unlikely. In experimentally induced brain tumors in animals, Arnold and Zimmerman[2,46,47] found several instances of mixed sarcoma and malignant glioma and some of these appeared to be intermingled. However, on transplantation each of the tumors grew separately. The intimate admixture of the two neoplasms in the metastatic lesion in this case can be best explained if one assumes that the sarcoma continues to be newly formed in situ from the same structure from which it originated in the primary site and is not a simple metastasis. There is morphologic evidence which strongly suggests that the sarcoma in this case arises from the periphery of blood vessels in the pulmonary tumor. That this does not represent invasion of the vessel by tumor can be seen by comparing these with a vein which is clearly being invaded (Figs. 6A-6D, 7A-7D, and 8). This process appears to be qualitatively similar to the formation of sarcoma cells from proliferating capillaries in Kaposi's sarcoma.

Craniotomy is generally considered to be the inciting factor in producing extraneural metastases of primary brain tumors. The operative procedure often leads to direct extracranial extension of the tumor and gives access to blood vessels and lymphatic channels. In the case under consideration five months elapsed between craniotomy and the first roentgenographic appearance of pulmonary metastasis. The maximum diameters (measured on successive chest roentgenograms) of the metastatic tumor were 2.0 cm. on April 3, 1956, then 3.0 cm. on May 17, 1956, and 3.5 cm. on June 26, 1956. An attempt was made to determine the growth rate with use of the method suggested by Collins.[7,8] The doubling times were, respectively, 25 and 58 days, with a mean value of extreme measurements of 34 days. According to the mean doubling time of 34 days, a metastatic embolus 100μ in diameter reached the lung about two years before the pulmonary metastasis was first noted or about one and one-half years before craniotomy. If one accepts the faster doubling rate of 25 days, it would place the onset of metastasis about a year before the craniotomy. The validity of any conclusions based on this concept depend on the

hypothesis of uniform and constant growth rate of tumors. In the present case the marked variation in the doubling time is not in keeping with previous observations of other tumors.[8] This inconsistency is probably attributable to the small number of measurements and the relatively short period of observation. It makes it difficult to reach an unequivocal conclusion as to the time of onset of the pulmonary metastasis. More complete information of this nature (including serial chest roentgenograms) in similar cases may well prove conclusive in establishing the relationship of operative procedures to extraneural metastases.

Summary

In a case of an intracerebral tumor there were two intermingled neoplasms, a glioblastoma multiforme and a spindle-cell sarcoma. In a lung metastasis a similar tumor was found. Reactive vascular hyperplasia was present in the cerebral tumor and in the lung metastasis. It was absent in the lung parenchyma outside the metastasis. It is suggested that the sarcoma was derived from the vascular hyperplasia in both the primary and the metastatic sites.

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Pathogenesis of Metastasis Formation Observed in Vivo in the Rabbit Ear Chamber

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After the initial appearance of neoplastic cells in the host, the formation of metastasis—the detachment of cancer cells and their successful lodgment and growth at new sites—is probably the most important phenomenon occurring during the natural history of cancer. Tumor dissemination by vascular embolism may be considered as occurring in three continuous stages: (1) *release* of cancer cells from the primary growth into blood or lymphatic channels; (2) *transport* of tumor cells via established vascular pathways, and (3) *lodgment, survival,* and *growth* of embolic cells at distant sites. Experimental studies [4,10,12,16] and histologic examination of the blood for cancer cells [8,26,27] indicate that malignant cells are almost constantly invading the vascular system in a number that far exceeds the total instances of metastasis. The enormous majority of embolic cells fail to become established; these cells perish within the blood stream.[3,15,30,32,34,37-40,42,47] Willis [42] concluded that experimental and autopsy evidence establishes that "tumor embolism is not metastasis," a concept first enunciated by Goldmann in 1897.[11]

From the histologic examination of both human and animal material, the principal cellular events associated with the development of intravascular cancer cells have been clearly described by several authors.[3,15,30,32,37,38,42] After the intravenous injection of carcinoma cells into rats, Warren and Gates [38] studied groups of animals killed at successive intervals and related in detail the consecutive histologic findings during the growth of cancer-cell emboli within the lung. In spite of these and many other detailed investigations, knowledge of the subject remains essentially descriptive. The underlying biochemical and biophysical reactions are unknown.

This communication describes in vivo observations on the fate of blood-borne cancer cells. These experiments required the designing of a special method for delivering cancer cells into minute vessels which could be repeatedly visualized in vivo. A detailed account of the technique finally adopted and a description of the lodgment, endothelial penetration, and growth of blood-borne cancer cells are presented.

Materials and Methods

Adult male Flemish-New Zealand stock rabbits* weighing approximately 3 kg. were used. The rabbit ear chamber technique originated by Sandison [29] and subsequently modified by Allison et al.[3] was used. The space between the central platform and the cover glass was machined within the limits of 20μ to 25μ.† Details of the method used for installing the chamber are described elsewhere.[46] ‡ The chambers were not used until tht blood vessels were fully mature (approximately six to eight weeks) and the tissue was free of cellular debris. A chamber was used for only one experiment.

The tumor used in these experiments was a squamous-cell carcinoma of rabbits, the V2 carcinoma § (Fig. 1), derived from the Shope virus papilloma.[17] This tumor is readily maintained in domestic rabbits. After intramuscular implantation,

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* Obtained from Eldridge Rabbitry, St. Louis.

† Ear chambers were prepared by Mr. O. G. Langner.

‡ Dr. F. Allison Jr., of the Department of Internal Medicine, University of Mississippi, Jackson, Miss., gave technical advice relating to the use of the chamber technique.

§ The V2 carcinoma was obtained from Dr. I. Zeidman, at the University of Pennsylvania, Philadelphia.

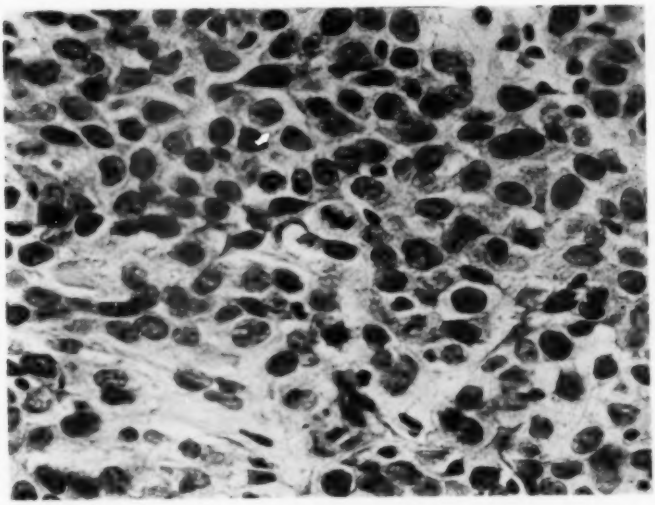


Fig. 1.—Section of V2 carcinoma, a poorly differentiated squamous carcinoma. Hematoxylin and eosin; ×470.

it metastasizes regularly to adjacent lymph nodes, occasionally to the lungs, and rarely elsewhere; in this behavior it resembles squamous-cell carcinoma in man.

Cancer-cell suspensions were prepared by mincing the solid tumor with scissors and then pressing it through a finely meshed tea strainer. The cells were diluted with 10 vol. of Morgan and Parker's Mixture 199.‖ This suspension was centrifuged at 300 rpm for five minutes. The resulting supernatant, used for injections, contained predominately single cells. Trypan blue was added immediately prior to injection to facilitate the identification of viable cells.

In order to follow the progress of the intravascular development of tumor emboli, unnarcotized rabbits were trained to sit quietly in the specially constructed rabbit box during microscopic observation of the chamber. The inverted microscope technique of Allison et al.[2] was employed. As shown in Figure 2, a special frame supported the microscope above and parallel to the rabbit box, with the stage facing downward. The ear of the rabbit was raised without tension to the level of the microscope. The ear chamber was clamped in place by a modification of Sanders and associates' chamber holder[28] (Fig. 3). The use of a calibrated mechanical stage made the repeated location of selected areas possible.

The light source was a 6 volt, 18 amp. ribbon filament microscope illuminator (Fig. 2). Heat was totally removed from the light by the use of both a heat-absorbing glass filter and a 10 cm. water cell.

Observations were recorded by means of 16 mm. black-and-white motion picture film. A film speed

‖ Obtained from Microbiological Associates, Bethesda, Md.

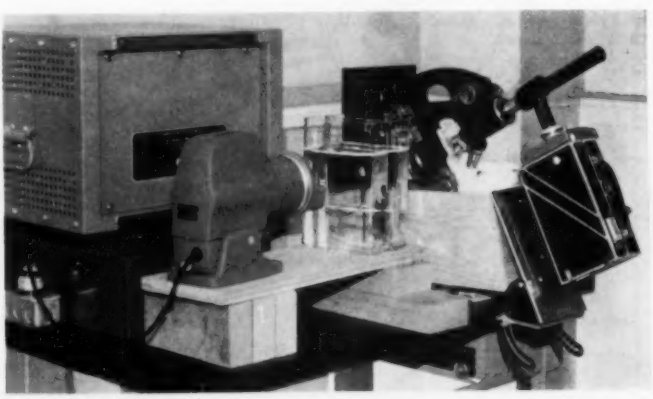


Fig. 2.—Photograph of microscope equipment, showing (left to right) constant D. C. power regulator, microscope illuminator, heat-absorbing filters, inverted research microscope, optical beam splitter, observation eyepiece, and 16 mm. motion picture camera used to study and photograph rabbit ear chamber.

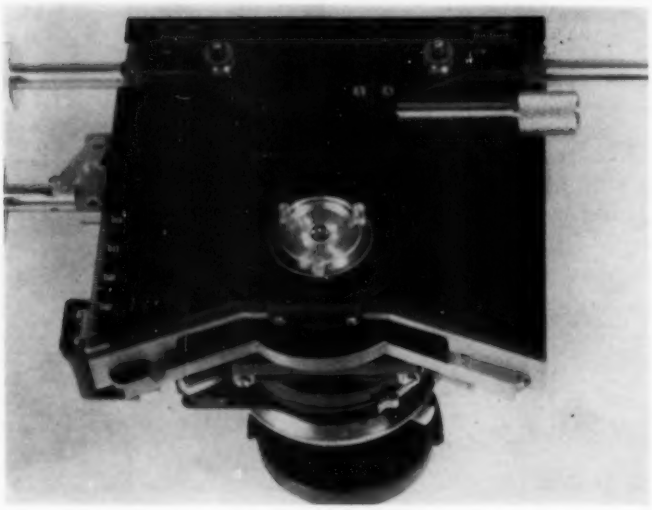


Fig. 3.—Modified mechanical stage with chamber holder.

of 64 frames per second permitted the slow-motion analysis of cellular events. Using this film speed, it was necessary to employ a direct-current constant voltage regulator as a power source (Fig. 2).

On the day of the experiment, the animal was placed in the box one hour before the start of the study to permit time for stabilization. No anesthesia was employed. The posterior portion of the rabbit ear over the central artery was epilated by a barium sulfide mixture. A 30-gauge needle, connected to a short length of polyethylene tubing and a 1 ml. syringe (Krogh-Keys syringe pipette) filled with freshly prepared tumor-cell suspension, was inserted in the central artery (Fig. 4). The point of the needle was advanced to within ¼ to ½ in. of the central platform of the ear chamber. After the needle and tubing were securely taped in position, the ear was gently placed in the mechanical stage, and the chamber was clamped in position for observation and photography (Fig. 2). Special modification of the lower border of the mechanical stage, as shown (Fig. 3), allows the ear chamber, needle, and tubing to be placed easily on the microscope stage.

Approximately 0.2 to 0.3 ml. of the tumor-cell suspension was slowly injected while observations were made. To avoid traumatic injury to the capillaries, it is imperative that the infusion be pro-

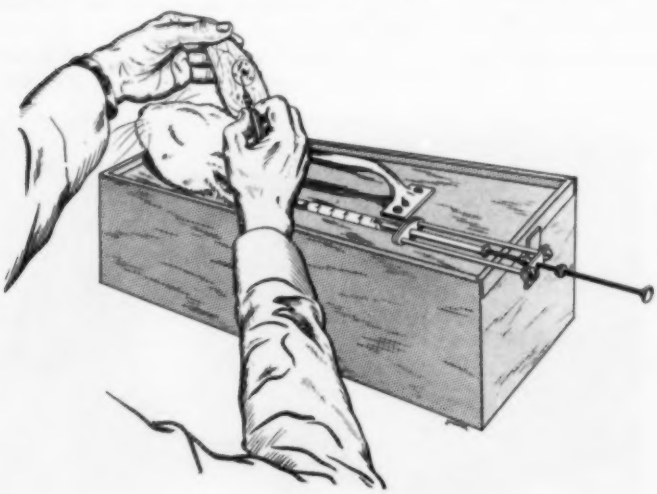


Fig. 4.—Diagram of technique for the slow injection of cancer cells into the auricular artery proximal to the ear chamber.

longed for 10 to 15 minutes. After completion of the injection, the needle was removed.

Results

When the experimental conditions were rigorously controlled, as outlined previously, the following microscopic observations were recorded during three typical experiments.

EXPERIMENT 1.—Two blue-stained cells passed rapidly from an arteriole to a minute capillary, where they adhered firmly to the endothelium. Before injection, normal blood flow had been observed in this particular capillary. After lodgment of the blue-stained cells, flow ceased for about eight minutes. At 10 minutes, the capillary dilated to a diameter two to three times the size of the blue-stained cells and flow through this vessel was brisk. Leukocytes rolled along the endothelium in their characteristic fashion. Between 20 to 30 minutes after the injection, thrombus developed around the dye-stained cells and slowly obstructed flow in the vessel. This thrombus appeared to contain fibrin-like material, platelets, and a few entrapped leukocytes. By the 12th hour, the thrombus had disappeared and flow through the capillary returned; yet the dye-stained cells, surrounded by a trace of fibrin-like material and a few leukocytes, remained firmly adherent to the endothelium. Between the 18th and the 24th hour, this portion of the capillary again thrombosed. Between 24 and 48 hours, the dye-stained cells were surrounded by the thrombus. The endothelium was indistinct and coated with leukocytes. By 48 hours, the dye-stained cells lost most of their coloration and increased in number, and penetration of the endothelium occurred. About eight large tumor-like cells, some containing dye, and several leukocytes were present in the capillary and extended into the perivascular connective tissue. This mass progressively increased in size during subsequent observations.

EXPERIMENT 2.—Five minutes after the infusion of dye-stained carcinoma cells was begun, a capillary was completely filled by a thrombus containing a large blue-stained cell mass, several individual stained cells, and a number of unstained cells, platelets, and rare leukocytes. The endothelium was somewhat indistinct. At 20 minutes, the dye-stained cell clump was securely enmeshed by thrombus and a few leukocytes were seen adjacent to the dye-stained intravascular embolus. The endothelium surrounding the larger embolus-thrombus was poorly defined. During subsequent hours, the individual dye-stained cells were dislodged as the surrounding thrombus fragmented, and flow resumed through this portion of the capillary.

By the ninth hour, brisk flow was observed in adjacent capillaries (Figs. 5 and

Fig. 5.—Stained cells at nine hours entrapped in thrombus. The surrounding endothelium was poorly defined. Leukocytes had accumulated along the altered endothelium. (Figs. 5-40 are enlargements and diagrams of single frames from 16 mm. cinemicrophotographs. The events are much more obvious when the entire motion picture is reviewed.)

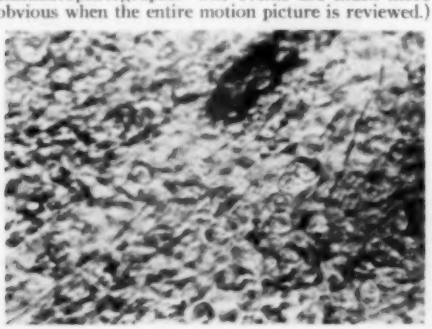
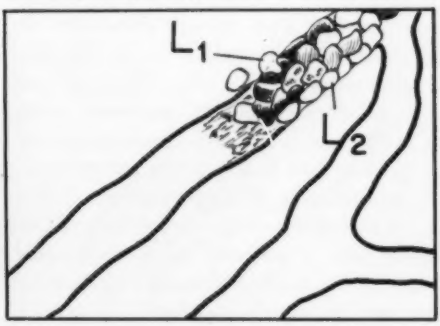
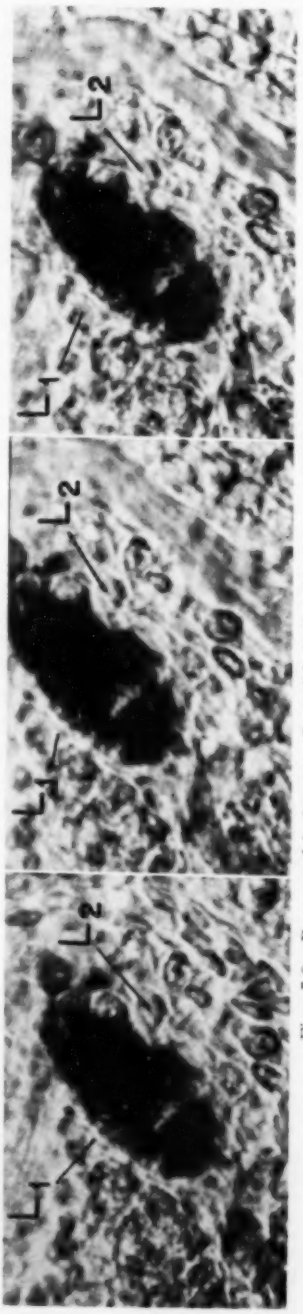


Fig. 6.—Diagram of Figure 5 showing stained cells, leukocytes, and thrombus. Flow was observed in adjacent capillaries. Leukocytes (L_1 and L_2) wandered over the areas of endothelial injury before emigrating through the vessel wall (Figs. 7-9).



6); the dye-stained cell mass was surrounded by a firm thrombus. The endothelium to which the dye-stained embolus was attached appeared to be poorly defined. Leukocytes migrated down the static vessel to the areas of endothelial injury at the site of the thrombus which contained the dye-stained cells. The leukocytes then began to migrate at random over the endothelial surface. It was not possible in advance to predict the precise focus of leukocytic penetration, since at no time were there visible endothelial stomata. Once the leukocyte had found a suitable spot for penetration, the process of diapedesis began. It usually required 3 to 15 minutes for completion (Figs. 7-9). After the leukocyte had passed through the vessel wall, a defect in the endothelium seemed to exist, for not infrequently one or more additional leukocytes would follow exactly the same route from the vascular lumen to the perivascular connective tissue. Once they had entered the perivascular connective tissue, leukocytes appeared to move at random. Perivascular histiocytes and erythrocytes did not appear to be involved in the cellular reaction. In every instance in this and subsequent experiments where motion picture records were available for analysis of endothelial penetration, tumor-cell penetration of the endothelium followed leukocytic diapedesis and occurred through the endothelial defect produced by the leukocytes.

At 12 hours, the endothelium surrounding the dye-stained embolus was fragmented, and both leukocytes and dye-stained cells had migrated through endothelial defects and accumulated in the perivascular connective tissue (Fig. 10). The emigration of dye-stained cells and occasional leukocytes into the perivascular connective tissue progressed. At 24 hours, the extravascular tissue contained a cellular infiltrate composed of leukocytes and tumor-like cells, of which only a minority were dye-stained. The primary capillary remained thrombosed and was eventually destroyed by cellular



Figs. 7-9.—Passage of leukocytes (L_1 and L_2) through the vessel wall. Total sequence was approximately 12 minutes. L_1 reached the extravascular tissue by the end of the sequence; L_2 passed through the wall more slowly.

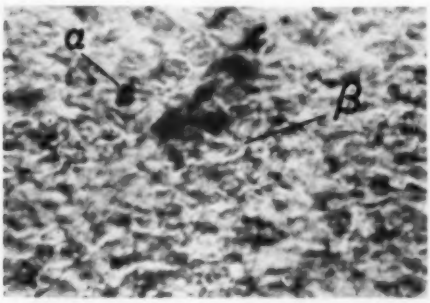


Figure 10

Fig. 10.—Extravascular accumulation (α and β) of leukocytes and tumor-like cells at 12 hours.

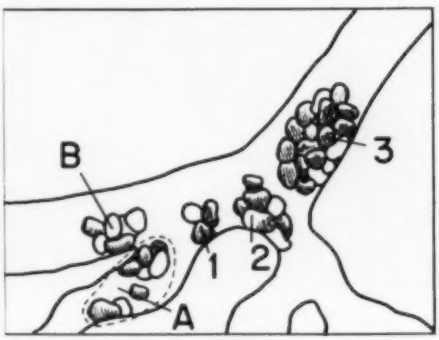


Figure 11

Fig. 11.—Diagram of capillaries showing initial location of two dye-stained emboli which failed to survive (Areas A and B) and of three separate emboli which grew (Areas 1, 2, and 3).

growth within it and in the perivascular connective tissue.

EXPERIMENT 3.—In this experiment, a single capillary bed was observed (Fig. 11), and a serial cinephotomicrographic record was obtained for detailed analysis. This 2600 ft. film depicts the failure of two dye-stained emboli (designated as Areas A and B), and the survival and growth of three separate cancer emboli (designated as Areas 1, 2, and 3). The principal cellular events observed in these five areas will be described separately.

Area A: Initially, two blue-stained cells were enmeshed in a loose "clot" composed of leukocytes and erythrocytes. Fibrin-like material and platelets could not be distinguished. The endothelium was clearly outlined at all times. From the earliest observations (Figs. 12 and 13), this intravascular mass was loosely formed and evidenced a slow to-and-fro movement. Microscopically, the dye-stained cells in this area were altogether identical with those observed in Areas 1, 2, and 3.

By the end of the first hour, the loose "clot" was breaking up, and there was appreciable ebb and flow within the vessel. The stained cells appeared loosely adherent to the endothelium. At two hours, there was definite pulsatile flow. One of the dye-stained cells became detached and was carried immediately into the adjacent venules. Between the second and third hour, the remaining dye-stained cell was loosened and carried swiftly into the venous channels.

Fig. 12.—Dye-stained emboli in Area A at 30 minutes. On the motion picture, these cells were observed to be loosely attached and surrounded by a loose "clot."

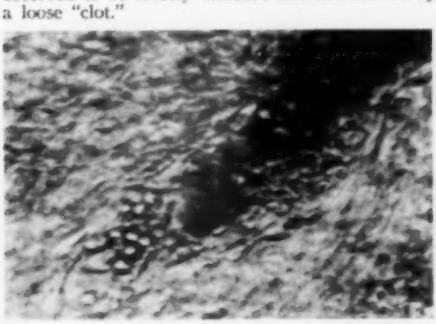
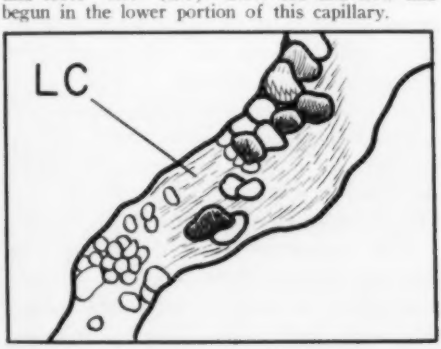


Fig. 13.—Diagram of Figure 12, showing emboli and loose "clot" (LC). Slow ebb and flow had begun in the lower portion of this capillary.



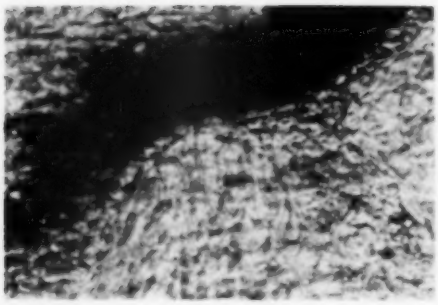


Figure 14

Fig. 14.—Emboli at 30 minutes surrounded by thrombus.

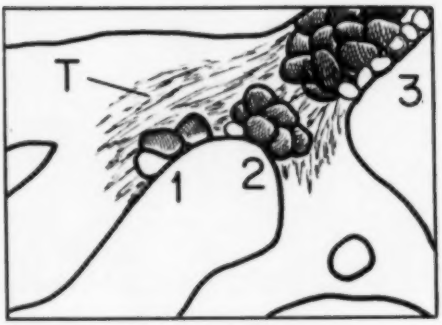


Figure 15

Fig. 15.—Diagram of Figure 14, showing location of emboli designated as Areas 1, 2, and 3. These cells were firmly adherent to the endothelium and enmeshed in thrombus (*T*). Note the accumulation of leukocytes along the altered endothelium subjacent to Area 3.

The precise nature or eventual fate of these two dye-stained cells is unknown.

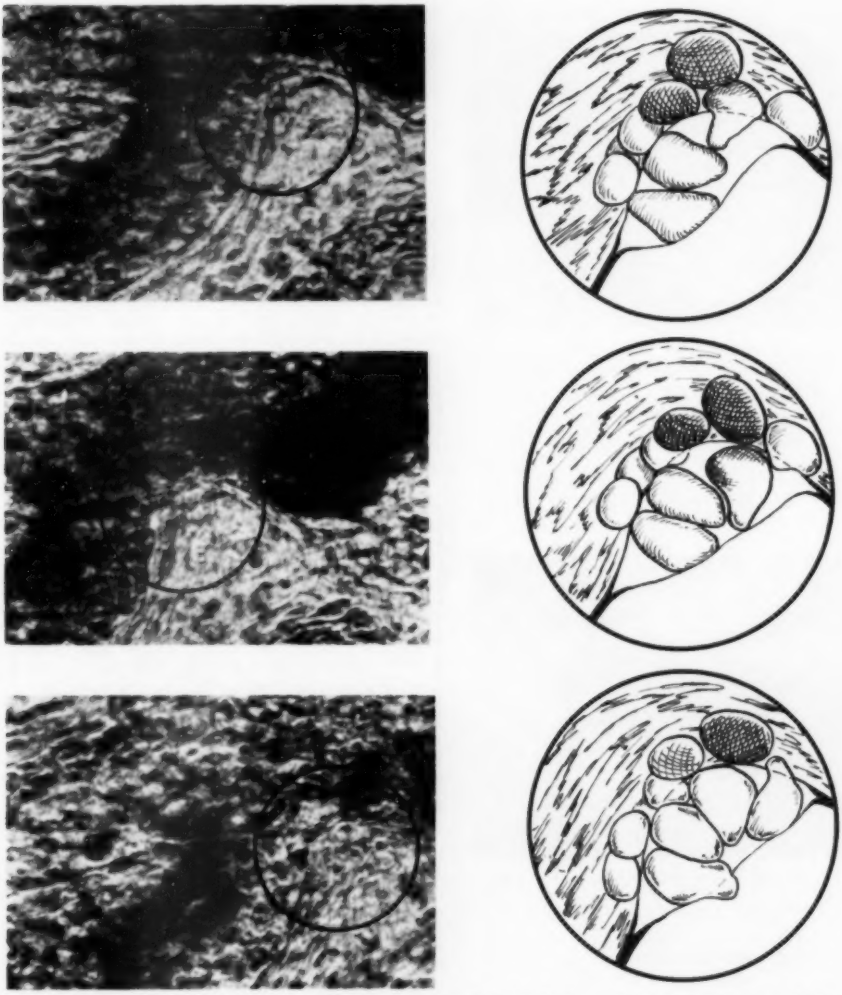
Area B: Observations of this embolus were essentially similar to those described for Area A. Two dye-stained cells and three or four similar unstained cells, histologically indistinguishable from the cells observed in Areas 1, 2, and 3, were loosely adherent to the endothelium and surrounded in a loose "clot." No fibrin-like material was observed, and the endothelium was consistently sharply defined. By the end of the first hour, slow ebb and flow of normal blood cells was seen in the vessel, and the embolized cells were loosely adherent to the endothelium. During the second hour, both of the dye-stained cells were dislodged and swept away by the vigorous pulsatile flow. Similar unstained cells persisted in this area for 18 hours, when they also were dislodged. During this entire period, there was no endothelial alteration, and following their detachment there was no leukocytic sticking.

Area 1: Initially, several dye-stained cells were adherent to the endothelium. By 30 minutes, these cells had been completely enmeshed in a firm clot (Figs. 14 and 15). The endothelium could be clearly defined.

After two hours, most of the thrombus was loosened and there was slow ebb and flow within the vessel. Two lightly stained cells were firmly adherent to the endothelium, which was somewhat indistinct. Several leukocytes emigrated and accumulated on the endothelium underlying the stained cells. Blood flow was slow above this area.

By three hours, the process of diapedesis had begun. This was the only instance observed in which it appeared that leukocytes were detained in the perivascular fibrous sheath for a short while after emigration (see Zweifach).[48] Two tumor-like cells subsequently penetrated the endothelial defect at this site; they also appeared to be detained temporarily within the perivascular fibrous sheath (Figs. 16-23). During the fourth hour, these tumor-like cells increased in number (Figs. 24 and 25). At 24 hours, subendothelial tumor-like cells were completely covered by a distinct glistening endothelial membrane. This focus protruded slightly into the vessel lumen, and leukocytes rolled without interruption over this endothelium. Within 30 hours, this subendothelial growth had increased to at least six cells, which had infiltrated the connective tissue (Figs. 26-28). At 42 hours, the subendothelial growth had extended deeper into the extravascular tissue. The covering endothelium was less distinct. Despite the rapid blood flow over this growth area, occasional brief leukocytic adherence was observed.

Area 2: Immediately after injection, several dye-stained cells were firmly adherent



Figs. 16-21.—Leukocytes at three hours passed through the endothelium and were detained temporarily within the perivascular fibrous sheath. Total sequence was approximately 20 minutes. A leukocyte (Figs. 22 and 23) finally penetrated the perivascular fibrous sheath.

to the lower endothelial border of this capillary. By 15 minutes, these stained cells were enmeshed in thrombus containing a small amount of fibrin-like material, platelets, rare leukocytes, and erythrocytes (Figs. 29 and 30). The underlying endothelium was sharply defined. Approximately one-half the capillary lumen was gradually filled by the slow extension of this thrombus; slow blood flow was observed in the remaining channel.

At 30 minutes, the vessel lumen was completely occluded by firm thrombus (Figs. 14 and 15). Within 50 minutes, the endothelium to which the stained cells were adherent lost its clear definition. Endothelium on either side of this site was sharply defined despite the presence of overlying thrombus.

During the second hour, the endothelium subjacent to the stained cells was notably indistinct. Leukocytes accumulated and

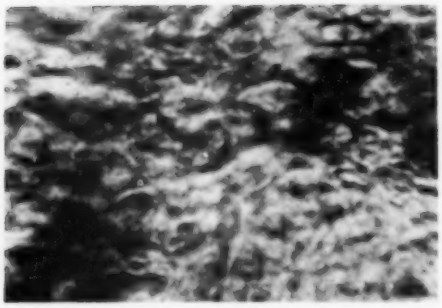


Fig. 22.—Two leukocytes almost completely penetrated the perivascular fibrous sheath at three and one-half hours.

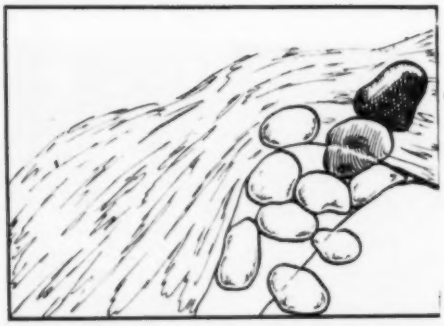


Fig. 23.—Sketch of Figure 22 showing two leukocytes which had passed almost completely through the perivascular fibrous sheath, and several leukocytes which had recently penetrated the endothelium, but were detained temporarily within the perivascular fibrous sheath. Two stained tumor-like cells were noted; one of these commenced emigration through the endothelial defect where previously leukocytes had passed through the vessel wall.

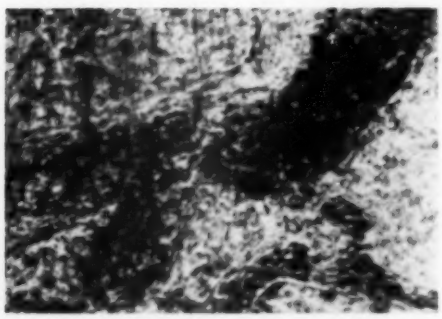


Fig. 24.—Capillaries at four hours.

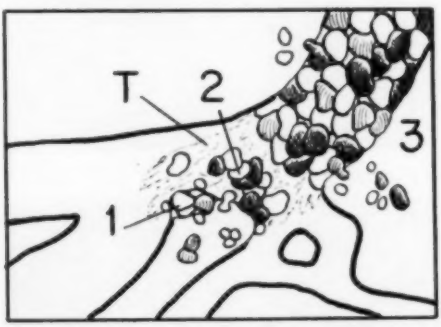


Fig. 25.—Diagram of Figure 24 showing Areas 1, 2, and 3 surrounded by thrombus (*T*). Endothelial penetration by leukocytes and tumor-like cells continued. Several large stained cells were noted at this time in the perivascular connective tissue. At the sites of penetration, the endothelium was fragmented.

Fig. 26.—Capillaries at 30 hours, showing extensive growth in perivascular connective tissue. Sites 1 and 2 are shown at higher magnification in Figures 28 and 37.

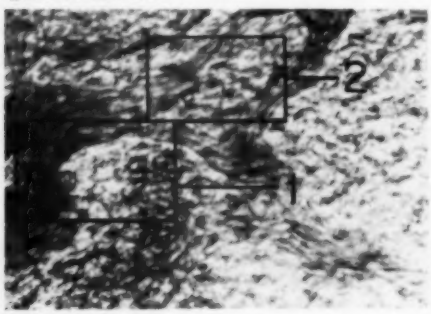
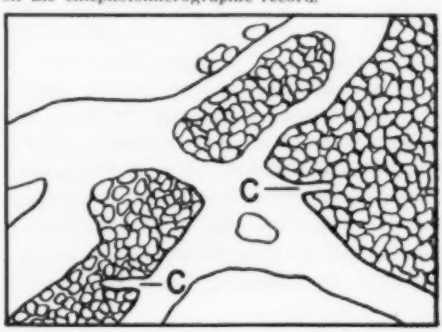


Fig. 27.—Diagram of Figure 26. Two-minute newly-formed capillaries (*c*) extended into connective tissue growth sites. Slow movement of blood cells into these capillary buds was observed on the cinephotomicrographic record.



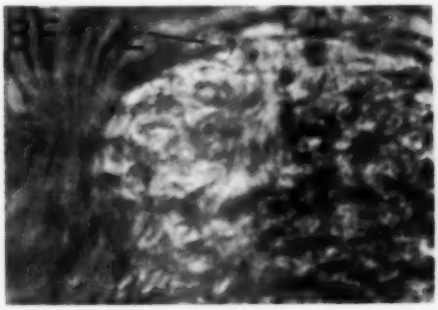


Figure 28

Fig. 28.—High power magnification of Site 1 from Figure 26, showing subendothelial growth from tumor emboli in Area 1. Brisk flow (*BF*) was seen in capillary lumen, and occasional leukocytes (*L*) stuck momentarily to the covering endothelium.

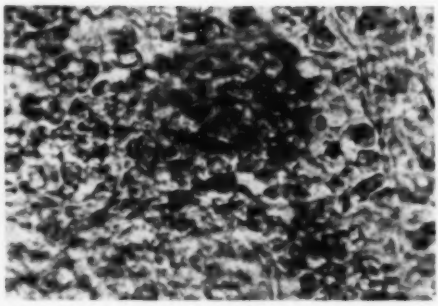


Figure 29

Fig. 29.—Emboli in Area 2 at 15 minutes. Stained cells were enmeshed in a thrombus. The underlying endothelium was sharply defined.

were eventually securely attached to the injured endothelium. Within the next 15 minutes, three leukocytes migrated through the altered endothelium into the connective tissue. A slow flow of blood cells appeared in the upper portion of this capillary after the progressive lysis of part of the thrombus. The stained cells on the lower border were consistently enmeshed in a firm thrombus, and around this mass several leukocytes migrated.

Within two and one-half hours, one of the stained cells migrated through the endothelial defect produced by the leukocytes. During the next few minutes, several leukocytes and tumor-like cells migrated through the endothelial defect and into the extravascular tissue. By three hours, the connective tissue contained at least six tumor-like cells (Fig. 18). Subsequently, a continuous slow migration of leukocytes and tumor-like cells into the connective tissue was apparent. Although the number of cells within the connective tissue increased, it was not possible to determine precisely how many were dividing tumor cells.

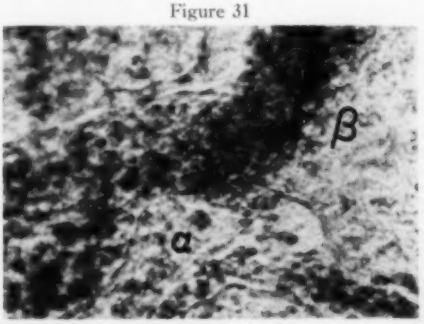
Within six hours, the majority of the intravascular tumor-like cells had lost their dye and migrated into the connective tissue, producing a very considerable tissue infiltrate (Fig. 31). At the site of cellular penetration, endothelium cannot be distin-

Fig. 30.—Diagram of Figure 29, showing stained cells enmeshed in a thrombus (*T*) containing fibrin-like material, platelets (*P*), rare leukocytes, and erythrocytes. Slow flow of blood cells was noted in the lumen about the embolus.

Figure 30

Fig. 31.—At six hours, the connective tissue infiltrate originating from emboli in Areas *2* and *3* is indicated by α and β, respectively.

Figure 31



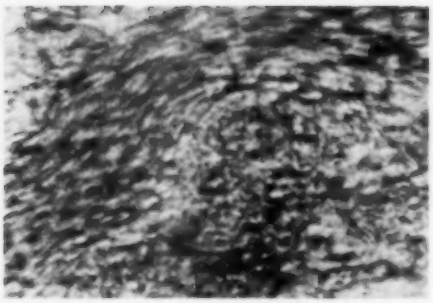


Figure 32

Fig. 32.—Extensive connective tissue growth from emboli (Areas 1 and 2) at 57 hours.

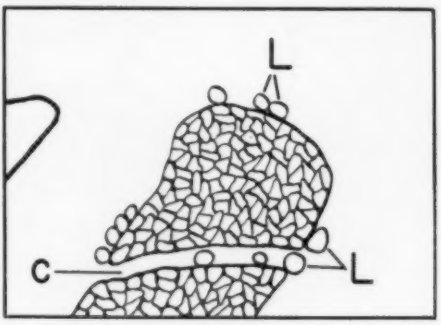


Figure 33

Fig. 33.—Diagram of Figure 32 showing leukocytes (*L*) adhering to endothelium. Flow in the initial capillaries was brisk. On the motion picture film, slow flow and leukocytic adherence were noted in the newly-formed capillary (*c*), which now extended completely through the tumor.

guished. A small number of faintly outlined tumor-like cells and occasional leukocytes were lodged within the firm thrombus which lined the lower portion of the parent vessel. Slow flow was observed in the upper half of this capillary. At 12 hours, the enlarging connective tissue cell mass contained a considerable variety of cell sizes and shapes; only three or four of these cells retained dye.

By 24 hours, growth in the extravascular connective tissue was more extensive. Of extraordinary interest at this time was the endothelialization of the penetration site. Leukocytes rolled slowly over this newly formed endothelium (Figs. 26 and 27). At this time, a minute capillary bud was first apparent. This ingrowth arose from a predestined to penetrate the altered vessel wall (see Figs. 35 and 36) was noted.

existing capillary and slowly extended from the periphery into the area of tumor growth (Fig. 27).

After 57 hours, this newly formed capillary had extended completely through the tumor (Figs. 32 and 33). Flow within this capillary was slow, and its endothelium was very poorly outlined, as, indeed, poor definition of capillary endothelium within or about growing tumors was frequently the case. This morphologic difference of intra- or peritumoral endothelium was often, though not invariably, associated with slight to appreciable leukocytic adherence.

After three days, the laterally expanding tumor obscured and ultimately destroyed these vessels.

Fig. 34.—Stained cells in Area 3 at two hours. Tumor-like cells were firmly enmeshed in thrombus. Leukocytes accumulated and wandered over the lower endothelial surface. One leukocyte (*L*)

Figure 34

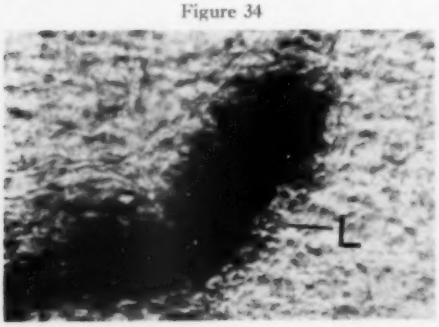
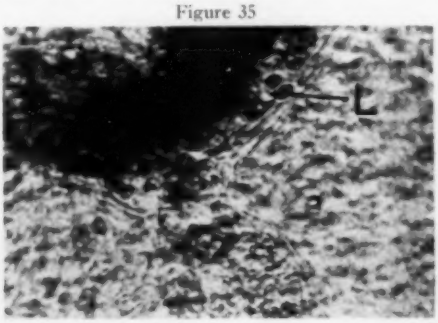


Fig. 35.—Partial endothelial penetration of leukocyte (*L*) at two and one-quarter hours.

Figure 35



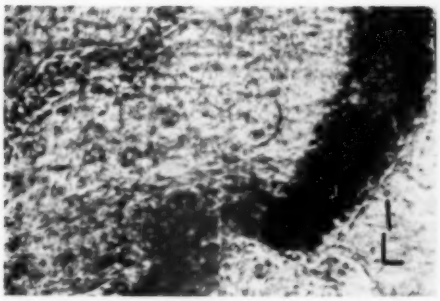


Fig. 36.—Approximately 10 minutes later, leukocyte (*L*) had reached the perivascular connective tissue and produced residual endothelial defect through which a stained tumor-like cell moved through the vessel wall.

Area 3: As observed in Areas 1 and 2, dye-stained cells became firmly adherent to normal-appearing capillary endothelium. During the first 30 minutes, they were slowly surrounded by a firm thrombus containing fibrin-like masses, platelets, and a few leukocytes (Figs. 14 and 15). The lower border of the endothelium was indistinct. Leukocytes migrated down the static vessel to the site of endothelial injury subjacent to the stained cells enmeshed in thrombus. Within two hours, numerous leukocytes migrated at random over the altered endothelial surface (Fig. 34). Beginning at two and one-quarter hours, a leukocyte penetrated through the altered endothelium (Fig. 35) and into the connective tissue (Fig. 36). At two and one-half hours, a tumor-like cell elongated and passed through the endothelial defect produced by leukocytic diapedesis.

During the third hour, the site of endothelial penetration enlarged, and a number of tumor-like cells and leukocytes escaped into the connective tissue. By four hours, there was a variety of cells, some unmistakably leukocytes and others resembling tumor cells both with and without stain, observed at the site of the endothelial defect and in the subjacent connective tissue.

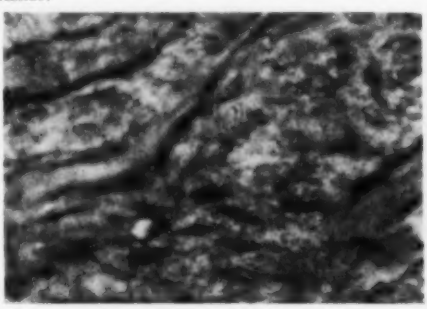
During the fourth hour, the upper endothelial border gradually lost its definition, and subsequently leukocytes accumulated along this surface (Figs. 24 and 25). Within a brief period, a leukocyte penetrated this surface and entered the connective tissue. Slow connective tissue infiltration by leukocytes and tumor-like cells occurred through this endothelial defect, although at a considerably slower rate than that observed along the lower capillary surface.

At six hours, the most prominent connective tissue infiltrate was subjacent to the site of earlier endothelial penetration. A number of tumor-like cells, several containing dye (at least two or three), were recognizable among these cells. By this time, part of the intravascular thrombus had been loosened, and there was a very slow movement of blood cells within this vessel. The dye-stained cells remaining within the vessel were firmly adherent to the endothelium.

By 12 hours, a marked increase in perivascular tissue cellularity was observed both above and below the parent vessel. Histologically, many of these resembled tumor cells, and a few contained stain. The endothelium in the two sites of penetration remained indistinct. At 18 hours, endothelial penetration and connective tissue infiltration were more extensive. Within the vessel proper, an increased number of tumor-like cells was apparent; these probably resulted from intravascular growth. Slow flow of blood cells continued within the vessel lumen.

Between 24 and 30 hours, the upper and lower areas of endothelial penetration had been covered by endothelial proliferation

Fig. 37.—High-power magnification of Site 2, Figure 26, showing the endothelialized intravascular tumor.



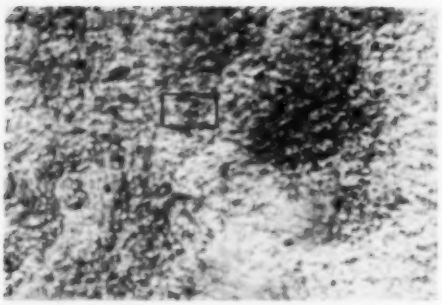


Fig. 38.—Capillaries and connective tissue growth at 42 hours. Details of the marked area are shown in Figure 40.

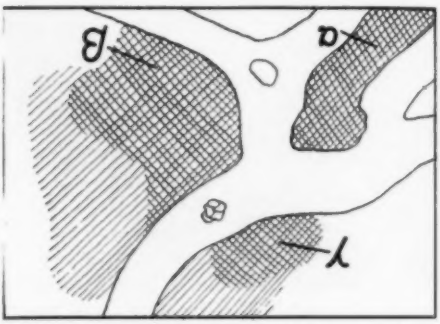
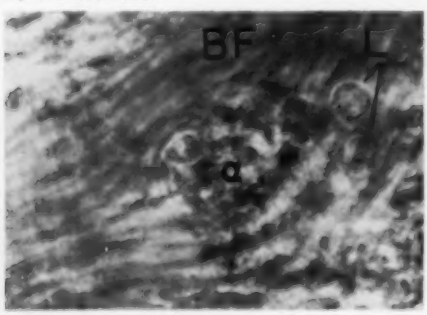


Fig. 39.—Diagram of Figure 38, showing dense connective tissue growth (α, β, γ).

(Figs. 26 and 27). Intravascular tumor-like cells, several still containing minute amounts of stain, were endothelialized (Figs. 26, 27, and 37). Except for momentary sticking of leukocytes as they passed over the sites of endothelial regrowth, the blood flow was relatively brisk. The extravascular tissue growths above and below the vessel have increased in size and cellularity. At this time, vascularization of the growing tumor areas began. A minute capillary ingrowth arose from a preexisting normal capillary and extended a short distance into the connective tissue growth (Fig. 27).

After 30 hours, a steady increase in the number of cells in the intravascular endothelialized site had occurred; within 42 hours, these tumor cells extended into the connective tissue both above and below the vessel (Figs. 38 and 39). Although its endothelium was intact, it could not be visualized as clearly as heretofore, and moderate leukocytic adherence was noted (Fig. 40).

Fig. 40.—High-power magnification of intravascular endothelialized growth (marked in Fig. 38), now considerably smaller after its extension above and below the vessel. Although blood flow was brisk (*BF*), moderate leukocytic adherence (*L*) was observed.



By 57 hours, growth within the connective tissue below the primary vessel was extremely cellular; newly formed capillaries extended through its center. The endothelium of capillaries within the tumor was indistinct, and slow flow was not infrequently obstructed by a leukocyte adherent to the endothelium.

During the third and fourth days, progressive peripheral tumor growth continued; this was accompanied by the gradual obstruction and eventual obliteration of preexisting and newly formed vessels.

Concluding Observation on Areas 1, 2, and 3: Between the third and fifth days, these individual areas of growth merged and became too cellular for detailed histologic study. At all times in our experience, once the tumor reached the connective tissue, it extended randomly in all directions; no histiocytic response or encapsulation occurred. The vessels within the central zone of the tumor were first obstructed and then obliterated. The rapid growth in the shallow depth of the chamber (20μ to 25μ) and vascular destruction both resulted in extensive central necrosis. The tumor, however, exhibited progressive lateral growth, and abundant new vascular anastomoses grew centrifugally.

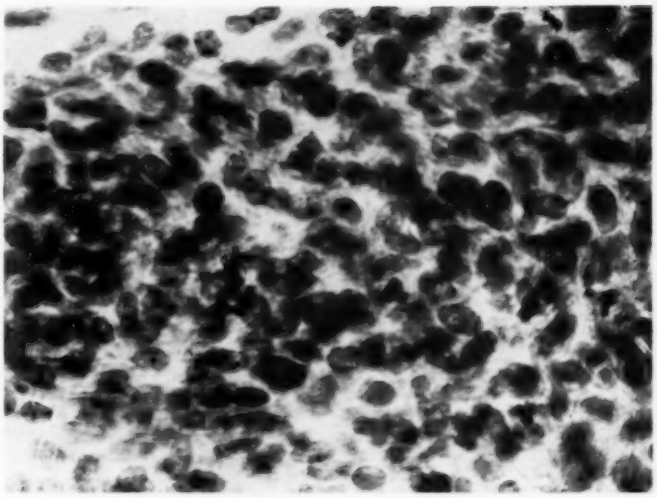


Fig. 41.—Section of V2 carcinoma metastasis in rabbit ear chamber, Experiment 3. Compare with Figure 1, V2 carcinoma following intramuscular inoculation. Hematoxylin and eosin; × 470.

Repeated observation of vessels outside the areas of tumor growth failed consistently to reveal intravascular sludging or thromboembolism described by previous investigators.[22]

By 13 days, the tumor was 3 mm. in diameter, the majority of the tumor was necrotic, and a narrow peripheral zone (varying in thickness between 100μ and 200μ) was extensively vascularized by newly formed capillaries. Within the peripheral zone of the tumor, capillaries were of normal size, although their endothelial surfaces were characteristically poorly defined and exhibited a general tendency for leukocytic adherence. In contrast, the vessels in the connective tissue adjacent to the tumor had sharply defined endothelium along which the leukocytes rolled in their normal fashion.

The rabbit was killed on the 13th day, and the chamber and visceral organs were fixed in Zenker's solution. Histologic examination of the growth within the ear chamber revealed the characteristic V2 carcinoma (Fig. 41). Microscopically, small metastases were found in other portions of the ear employed for this experiment and in the lungs.

Comment

The experiments reported in this paper constitute a new approach to the pathogenesis of metastasis formation, namely, direct microscopic examination of blood-borne cancer cells during their lodgment, endothelial penetration, and growth in the rabbit ear chamber. By this method it has been possible to follow the course of events step by step. The observations can be recorded by motion pictures, which serve as permanent records and are particularly valuable for an objective analysis of the cellular phenomenon and comparison of their rates of change.

Although at the present time reliable cytologic criteria cannot be established by which carcinoma cells may be categorically distinguished within the ear chamber, successive observations on their behavioral characteristics clearly reveal their malignancy: firm adherence to capillary endothelium, entrapment within a thrombus, endothelial penetration, progressive growth evoking the ingrowth of new capillaries, and ultimate formation of a gross tumor. Histologic examination of fixed and stained sections showed characteristic V2 carcinoma cells.

By using the trypan blue vital staining technique, it was possible to observe the passage of V2 carcinoma cells through the arterioles and into the capillaries where they lodged. Warren and Gates [38] observed that embolic cancer cells establish themselves in capillaries but only rarely in arterioles. In the rabbit ear chamber, successful metastasis formation has up to the present time been seen only within capillaries.

The site along the capillary endothelium to which the cancer cell might adhere could not be predicted. Capillary diameter was not a limiting factor, based on the firm adherence of cancer cells within vessels whose diameters were sometimes three times larger than the cancer cell and whose blood flow continued after tumor-cell lodgment. Additional observations are required before it can be stated by what mechanisms tumor cells may pass completely through capillary beds and how significant arteriovenous anastomoses might be in this regard.

Of the various cellular phenomena involved in metastasis formation, none is more fascinating to observe in vivo than the initial sticking of cancer cells to the vascular endothelium. The factors which account for its occurrence are at present unknown. The endothelial adherence by tumor cells appeared to be independent of leukocytic sticking, vasomotor activity, or capillary flow rate. In view of these considerations, and the consistent observation that the endothelium had appeared normal prior to lodgment, one gains the impression that the tumor cell from which a metastasis develops possesses a most extraordinary capability to attach itself to the endothelial surface. With the ear chamber technique, this phenomenon lends itself well to further investigation using a variety of procedures, including phase-contrast, ultraviolet, and electron microscopy, as well as histochemical stains.

After a latent period of only a few minutes, the formation of a thrombus was evident. This observation confirmed in vivo careful microscopic studies of fixed tissue sections, which showed that, in the earliest stages in the development of embolic tumor cells in both human and animal material, cancer cells are frequently enmeshed in intravascular thrombi.[3,15,30,32,37,38,42] In Areas A and B of Experiment 3, the failure of embolic tumor cells may be related to their inability to attach securely to the endothelium and evoke the formation of a surrounding clot. Heparin,[35,45] bishydroxycoumarin (Dicumarol),[45] or the fibrinolytic enzyme, plasmin,[6] administered prior to the intravenous inoculation of tumor cells, reduced the resulting number of lung tumors, and heparin [44] or bishydroxycoumarin [18] treatment of animals reduced the incidence of visceral metastases in animals bearing transplanted neoplasms. These observations indicated that the fixation of cancer cells within minute intravascular coagulae may be considered an essential process in the development of metastases from blood-borne cancer cells.

Recent studies have suggested that the clotting mechanism of the blood may be intimately involved in the sticking of leukocytes to the vascular endothelium.[2,48] The thromboplastic properties of many tissues, including certain cancer cells, are well recognized. In consideration of the V2 carcinoma, a tumor extraordinarily rich in thromboplastic material,[19] the ability of the carcinoma cell to become attached to normal endothelium and subsequently to be enmeshed in a thrombus may conceivably represent one of its distinctive characteristics.

In evaluating the mechanisms responsible for the endothelial adherence of cancer cells, the role of other factors considered important in leukocytic sticking warrants mention. These include vasodilation,[2,24] postulated changes in cellular surface charges brought on by "currents of injury," [31] and protoplasmic processes extending from the endothelial cells.[5]

The mechanism by which the V2 carcinoma cells penetrated the endothelium involved a complex sequence of events, which

were remarkably uniform when the complete cinephotomicrographic record was available. Within a period varying from 30 minutes to several hours, a progressive loss of endothelial definition occurred in the area immediately adjacent to the cancer cells and its thrombus. The precise etiology of this endothelial damage is unknown. Histamine-like substances, derived from the hydrolysis of tissue proteins,[20] and other amino acids and polypeptides [33] are known to be capable of causing endothelial damage and the typical vascular response of acute inflammation. Whether proteolytic enzymes released from the cancer cells diffused to the endothelium and produced this injury remains undetermined.

Within a relatively short period of time after endothelial damage, leukocytes emigrated down the static vessel and accumulated in the areas of endothelial injury. They migrated at random over the endothelial surface before penetrating the walls of the vessel. Numerous workers have observed that adherence to the vascular endothelium constitutes an essential step in the process of diapedesis of leukocytes.[2,5]

After one or more leukocytes had passed through the vessel wall, an endothelial defect resulted, through which cancer cells emigrated from the blood vessels into the connective tissues. In every instance, one or more leukocytes penetrated the endothelium in advance of the cancer cells. On two occasions, tumor cells have been observed outside of the vessel within three hours after their adherence to the endothelium; in one instance, penetration did not occur until 48 hours had elapsed.

As early as 1863, Virchow [36] described the ameboid motility of cancer cells. The V2 carcinoma grown in vitro moves at an average of approximately 0.7μ per minute.[9] Migration at this rate would readily explain the prompt passage of tumor cells through the endothelium and into the connective tissue.

Photomicrographs published by Iwasaki [15] and Warren and Gates [38] clearly show leukocytes within the thrombi which surrounded intravascular cancer cells, and a small number of leukocytes are visible in the perivascular connective tissue. Warren and Gates [38] reported that by six hours after intravenous injection of Walker 256 carcinoma cells, invasion and growth beyond the capillaries were evident. Their observations are in general agreement with the results obtained with use of the rabbit ear chamber technique, although with this technique the prompt accumulation of leukocytes and emigration of leukocytes and cancer cells from the vessel into the connective tissue is clearly evident.

The role of leukocytes, in general, and lymphocytes, in particular, in relation to malignant disease has been the subject of extensive research, especially by Murphy.[23] Recent observations by Humble and associates [13] warrant special consideration. Studying the interaction between lymphocytes and cancer cells in vitro, these authors report that lymphocytes showed a remarkable affinity for malignant cells, especially during mitosis. Leukocytes are suggested as being mobile sources of enzymes or metabolites which are particularly in demand by actively growing and dividing cells; such an association is considered reciprocally beneficial. Additional investigation is required to define the role played by the leukocytes in metastasis formation in the present experiments.

Schmidt,[32] Iwasaki,[15] and Saphir [30] described tumor cells which were covered by a thin layer of endothelium continuous with the intima. Endothelialization of tumor cells with subendothelial growth and subsequent connective tissue invasion was clearly confirmed in the present experiments. It appears that if attached tumor cells remain within the vessel for 24 hours and only partially invade the endothelium, they may be covered by endothelial proliferation. If all tumor cells migrate into the connective tissue, the endothelial defect may be repaired by normal-appearing endothelium. In either case, blood flow through the parent

vessel is maintained until it is occluded and destroyed by later tumor growth.

Many workers have drawn attention to the correlation between tumor growth and newly formed capillaries which originate from preexisting vessels.[1,14,21] Studying the growth of fragments of the Brown-Pearce carcinoma in the rabbit ear chamber, Ide and associates [14] noted blood vessel ingrowth around the tumor transplant as early as three days after transplantation. Using the mouse chamber technique, Algire and Chalkley [1] emphasized that tumor transplants elicited capillary growth as early as three days after implantation, whereas six days elapsed prior to the beginning of capillary proliferation in a wound. Studying the vascularization of omentum transplanted as autografts in a modified rabbit ear chamber, Williams [41] observed that new capillary growth from preexisting blood vessels began within 24 hours.

In the present experiments, capillary buds arising from preexisting vessels and entering the tumor were clearly visible within 24 hours. Subsequently, capillary proliferation continued, and at 57 hours the tumor contained a rich capillary supply. The mechanisms by which the malignant cell is able to provoke a continued vascular proliferation remain obscure.

Finally, brief mention should be made of the future uses of this technique. Of particular interest will be the use of this in vivo technique to investigate metastasis formation in altered states, such as during tumor growth at a remote site, after vascular injury produced by irradiation, infection, or trauma, and during treatment with drugs. Of practical importance might be observations after treatment with (1) factors known to enhance experimental blood-borne metastasis formation, such as cortisone,[25,44,45] stress,[45] or growth hormone,[43,44,45] and (2) substances reported to decrease metastasis formation, such as heparin,[35,44,45] bishydroxycoumarin,[44,45] or chemotherapeutic agents, e. g., nitrogen mustard,[7] employed for the prevention of tumor metastasis during surgery.

Summary

A special adaptation of the rabbit ear chamber technique has been devised to study and photograph in vivo, under high magnification, the intravascular behavior of cancer cells. Trypan blue-stained V2 carcinoma cells were injected slowly into the auricular artery of rabbits having vascularized ear chambers. Serial cinephotomicrographic records serve as a permanent documentation and are particularly valuable for an objective analysis of the cellular phenomenon and comparison of their rates of change.

Systematic observations of the fate of blood-borne cancer cells in the living animal may be summarized as follows:

1. Injected cancer cells passed rapidly from the arterioles into the capillary bed, where they became firmly adherent to the endothelium. The site along the capillary endothelium to which the cancer cell might adhere could not be predicted. The initial endothelial adherence by tumor cells appears to be independent of capillary diameter, leukocytic sticking, vasomotor activity, or rate of blood flow.

2. After a latent period of only a few minutes, intracapillary thrombus formation about the adherent cancer cells was first noted. These minute thrombi slowly extended to enmesh the cancer cells and obstruct blood flow. Subsequently, not infrequently part of this peritumoral thrombus was fragmented and dislodged, although cancer cells destined to penetrate the endothelium remained firmly adherent.

3. Within 30 minutes to several hours, the endothelium subjacent to the cancer cells was altered. Shortly thereafter, endothelial damage was apparent. Leukocytes migrated down the static vessel, accumulated in the areas of endothelial injury, and moved at random over the endothelial surface before emigrating through the capillary wall.

4. Penetrating leukocytes appeared to leave behind endothelial defects through which other leukocytes, and later cancer cells, emigrated. The earliest that tumor cells were observed to reach the perivascular

connective tissue was within three hours. Tumor growth in the extravascular tissue was progressive after six hours.

5. Tumor cells which had incompletely penetrated the endothelium were covered by endothelial proliferation within 24 hours, and flow through the parent vessel was re-established. Such subendothelial tumor cells subsequently grew and infiltrated the connective tissue.

6. Capillary buds arising from preexisting vessels and entering the tumor were clearly visible within 24 hours. Capillary proliferation was progressive, and by 57 hours, the tumor contained a rich capillary bed.

7. After the third day of continued lateral growth, the vessels within the central zone of the tumor were first obstructed and then obliterated. New vascular anastomoses grew centrifugally. Histologic examination of fixed and stained sections showed the characteristic V2 carcinoma.

The failure of embolic tumor cells to establish metastasis in the ear chamber may be related to their inability to attach securely to the endothelium and evoke the formation of a surrounding thrombus.

An outline is presented for the possible value of this new approach for the study of (*a*) the intimate mechanisms of lodgment, endothelial penetration, and growth of blood-borne cancer cells and (*b*) the prevention of hematogenous metastasis.

Sgt. E. R. Chevalier, Miss P. H. Mauk, and Miss E. L. Gallant gave technical assistance, Mr. J. D. Allred and Sgt. C. F. Curtis developed the cinephotomicrographic technique, and Major A. H. Doe and Mr. I. W. Richardson prepared the illustrations. Mr. F. F. Jasmer prepared the diagrams.

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Slide-Chamber Method to Measure Sensitivity of Cells to Toxic Agents

Application of the Method to Normal and Leukemic Human Lymphocytes

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The disease of chronic lymphocytic leukemia provides ideal conditions for the in vitro study of therapeutic agents. Patients with this disease are available in sufficient numbers in many hospitals and clinics. The leukemic cells can be readily obtained in adequate amounts and at frequent intervals without the inconveniences of biopsy or operative procedures. The test specimens are in a form to facilitate isolation and microscopic observation of the patient's cells. Adequate control specimens of homologous normal cells can be obtained without difficulty. In addition, the in vitro findings can be correlated with and evaluated by objective criteria of the patient's response to the same therapeutic agent.

A method is presented in this paper to determine the survival rates of normal and leukemic lymphocytes. The cytoxicity of a reagent to these cells can be measured quantitatively by this method.

Methods

Cellular Suspensions.—The method consists essentially of a modified form of tissue culture. Accordingly, the precautions and methods of tissue culture were followed as regards sterility, washing of glassware, and preparation of media.[4]

About 10 ml. of blood was obtained from a leukemic patient and transferred to a 15 ml. centrifuge tube which contained 0.2 ml. of a 0.2% solution of heparin (equal to 50 units) in distilled water. The tube of blood was placed in an upright position at 37 C to permit spontaneous sedimentation of the red blood cells but not of the leukocytes. The rapidity of sedimentation varied for different persons from 15 to 60 minutes. The turbid plasma was removed and contained, on microscopic examination, lymphocytes, platelets, and a relatively small number of erythrocytes and granulocytes. The success of the sedimentation method depended on individualization in the handling of each blood specimen without depending on any rigid time schedule. The plasma was centrifuged at 80× g for five minutes, and the supernatant with its platelets was removed completely. The precipitated cells were washed twice with Earle solution and resuspended in the final medium, which consisted of equal parts of TC 199 solution[10] (Difco Laboratories, Inc.) and human serum from a normal person with the same ABO blood group as the donor of the blood cells.

The cells in the suspension were counted by the unstained cell method described elsewhere.[17] To 0.2 ml. of suspension was added 0.8 ml. of an eosin solution (1:1000 in Tyrode solution); a drop of the mixture was placed in a hemocytometer, and the stained, unstained, and red blood cells were counted. The unstained cells were considered viable, and the stained cells, dead. The red blood cells could be readily differentiated from the unstained leukocytes by their dark contour and yellowish color. Granulocytes frequently flattened themselves on the hemocytometer and were sometimes difficult to see.

The slow sedimentation rate of normal blood necessitated the use of another method * for isolating normal lymphocytes. An attempt to follow the method given by Jago [9] was unsuccessful. Satisfactory results were obtained routinely by centrifuging normal heparinized blood for four successive times at 27× g for five-minute intervals with withdrawal of the plasma after each centrifugation. The cumulated plasma was centrifuged again for five minutes at 64× g and the resultant supernatant centrifuged at 110× g. The precipitated cells were washed twice with Earle

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* The method was developed with the collaboration of June Zerwekh.

solution containing 10% normal human serum. The cells were suspended in a mixture of equal parts of TC 199 and normal human serum of the same blood group as the cells. In one experiment, 10 ml. of blood yielded 4 ml. of final suspension, which contained in 1 cu. mm. approximately 1500 lymphocytes, 1500 erythrocytes, 200 other cells, and a small number of platelets.

Cellular suspensions were also made from lymph nodes removed for biopsy purposes. First the capsule was removed, and the node placed in a watch glass with about three times the volume of Earle solution. The red blood cells in the capsule were washed out with a saline solution to give a red blood cell suspension for determination of the blood group. The node itself was chopped up in Earle solution with a pair of fine sharp scissors. All nodes except those that were fibrotic yielded good cellular suspensions. The suspension was then passed through a Seitz filter fitted with an 80-mesh Monel metal wire screen to remove clumps of cells. The filtrate, which contained many isolated lymphocytes and a small number of other cells, was centrifuged at 80 g for five minutes. The precipitated cells were washed once and resuspended in 50% normal human serum. Unstained cell counts were made to determine the numbers of viable and dead cells and red blood cells. Usually about 25% of the lymphocytes were dead and stainable with eosin.

The suspension derived from blood or a lymph node was diluted with 50% normal human serum, to give about 1500 viable leukocytes per cubic millimeter. The test reagent in suitable dilutions was added to aliquots of the cellular suspension and the mixtures used for slide preparations. The pH of the diluted cellular suspension was 7.5 to 8.0, as determined with a Coleman pH electrometer equipped with a microchamber. The sterility of the suspension was routinely checked by inoculation of a brain-heart-infusion medium.

Slide Preparations.—A sterile cover glass, 50×75 mm., was covered with the top of a Petri dish to prevent contamination and evaporation during the preparation of the slide. In the center of the cover slip was placed a metal plate, 40×40×0.85 mm., with a central hole 26 mm. in diameter. A thin rectangle about 12×15 mm. was drawn with petrolatum on the cover glass inside the hole of the metal plate; 0.2 ml. of the cellular suspension was placed inside the petrolatum ring, which prevented excessive spreading or movement of the fluid. Another cover glass, 43×50 mm., was gently lowered on the drop of suspension. The drop filled the petrolatum ring and the space between the two cover slips. The preparation was sealed with freshly melted yellow wax (beeswax) and incubated at 37 C.

The cells in the preparation settled rapidly on the lower cover glass, and it was therefore necessary to use an inverted microscope with phase optical equipment for the observation and photography of the cells. The microscope was equipped with a phase, × 45, fluorite objective with N. A. 0.95, a binocular attachment with × 2 magnification and × 10 oculars. More recently, a × 95 objective was used to count cells. The inverted microscope was enclosed in an incubator so that the slide preparations could be maintained at 37 C during observation or time-lapse cinemicrography.

Criteria of Viable and Dead Lymphocytes.—The next step was to obtain a quantitative measure of the number of viable lymphocytes in the preparation. The criteria used to recognize a viable lymphocyte were based on preliminary observations of many slide preparations studied on successive days. These observations were supplemented by time-lapse cinemicrographic studies to compare the more or less static visual findings with the speeded up activity of the cells in the projected film.

The viable, i. e., the morphologically intact and unaltered, normal lymphocyte was round or had a broad anterior pseudopod and a posterior tail (Fig. 1). The nucleus was large, round, or oval, with prominent chromatin masses, some of which were attached to the nuclear wall. Some nuclei had large, gray, irregularly shaped nucleoli. The cytoplasm appeared sparse and dark gray, and frequently a few small granules or mitochondria could be seen. The cell and nuclear outlines were clearly visible, but the morphologic features and the contrast were somewhat soft and the cell and nuclear walls were not prominent. The viable lymphocyte showed slow oscillating movements which were conspicuous in time-lapse cinemicrographic studies but were barely perceptible on direct microscopic observation. The lymphocytes from many patients with chronic lymphocytic leukemia were fairly mature and similar in appearance to normal lymphocytes (Fig. 2). Immature cells were somewhat larger in size; chromatin masses were fewer in number, and nucleoli were larger and more numerous.

The differentiation of the lymphocyte from the granulocyte and monocyte has been described by several investigators [1,2,8] who have explored the use of phase microscopy in hematology. In this work the granulocyte was frequently recognized by its physiologic properties, such as motility, cytoplasmic

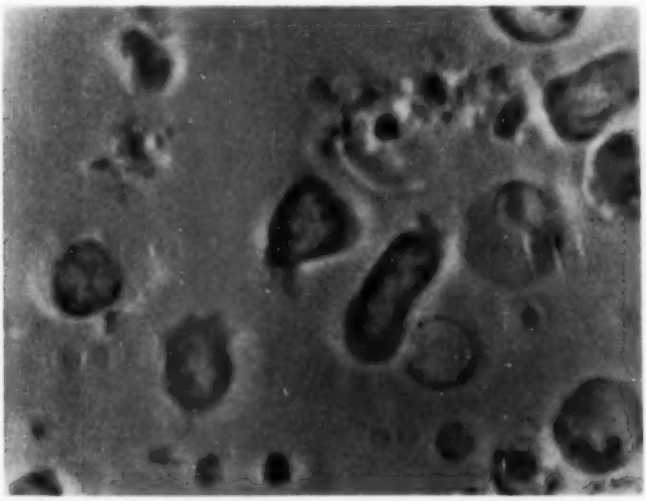

Fig. 1.—Normal human blood cells in a slide preparation incubated at 37 C for 5 days. Figures 1 to 4 are enlargements made from 16 mm. time-lapse cinemicrographic films. In this figure, five round or irregular shaped lymphocytes with coarsely granular nuclei are considered to be viable and in the projected film show considerable oscillatory movements. Two other cells with a few cytoplasmic granules but without nuclei are probably granulocytes which have undergone death and karolysis. Reduced about 10% from mag. × 1750.

streaming, and a tendency to flatten itself on the glass surface. Some granulocytes did not show any of these properties but were recognized by their relatively large size, their finely or coarsely granular appearance, and an obscure multilobed nucleus. The monocyte also had a tendency to flatten itself on the glass, with the formation of a peripheral undulating membrane. The monocyte was relatively large, and the nucleus did not have the coarse masses seen in the lymphocyte.

The dead or degenerating lymphocyte varied in appearance, depending on the type and dosage of the reagents used. Two types of cell death have been observed in this work. One is death associated with intranuclear vacuolation [12] and lobulation and terminating in a cell with a pyknotic nucleus with or without fragmentation of the nucleus (Fig. 3). The same type of death occurred in nonirradiated lymphocytes (Fig. 2). These changes are so drastic that this death can be readily recognized.

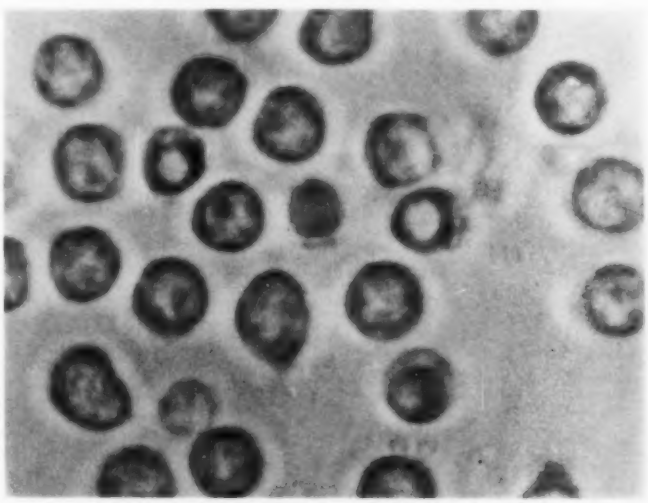

Fig. 2.—Lymphocytes from the blood of a patient with chronic lymphocytic leukemia. The slide preparation was incubated for 21.9 hours. Nearly all the cells have well-defined nuclei with chromatin and nucleolar masses and are considered to be viable lymphocytes. Two cells with large intranuclear vacuoles and chromatin rings and two other cells with large dark pyknotic nuclei are considered dead.

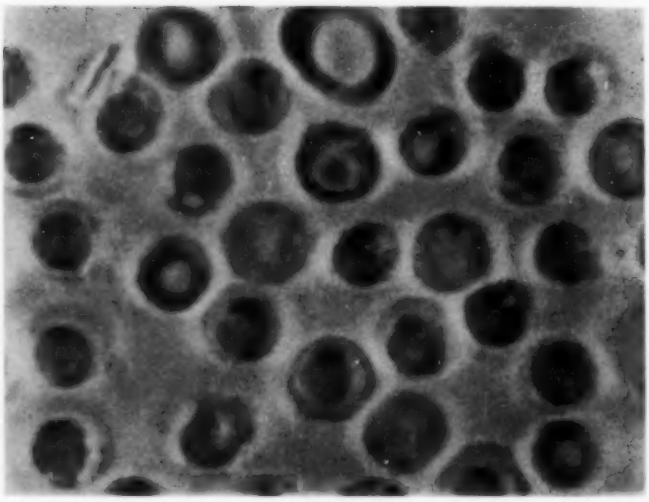

Fig. 3.—Leukemic lymphocytes irradiated with 1000 r and incubated for 11.2 hours. The control nonirradiated cells of this experiment are shown in Figure 2. Only one cell has retained its morphologic characteristics and is a viable lymphocyte. Four cells have intranuclear vacuoles and chromatin rings. Most of the cells have dark, structureless, pyknotic nuclei and a small amount of cytoplasm. In a few cells no nuclei can be made out, owing to lysis of the pyknotic nuclei, as can be seen in the projected cinemicrographic film. The large cell with a clear center is a biconcave macrocyte. In direct microscopic examination the biconcave erythrocytes can be readily differentiated from lymphocytes with intranuclear rings by color and other characteristics.

A second type of death has been called delayed fixation,[13] which is a more subtle change in the cell and which may be somewhat difficult to recognize during viable cell counts. This process usually caused the cell to round up and the nucleus to undergo minor changes, such as irregularities in shape or thickening of the nuclear wall and bilobed nuclei (Fig. 4). In time-lapse cinemicrographic films, the cell killed by fixation usually lacked rhythmic movements. The crucial feature of this type of death was that the fixed cells suffered postmortem changes, which included blurring or autolysis of the nucleus and edema of the cytoplasm. In a few hours, the nucleus appeared to be small, dark, and structureless or was completely absent from the cell. Suspen-

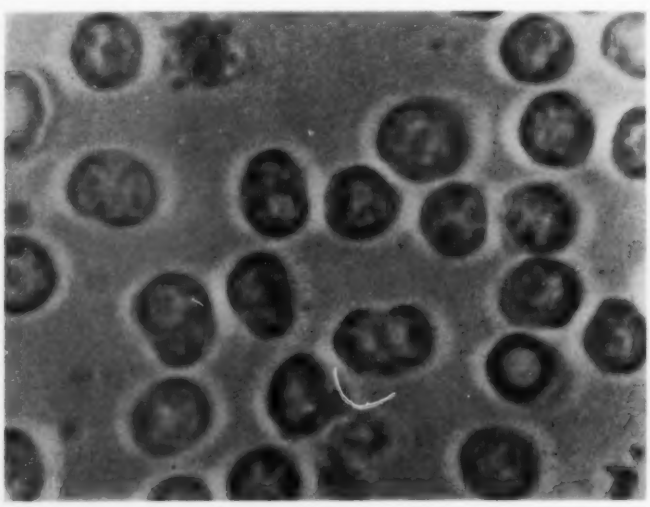

Fig. 4.—Leukemic lymphocytes, same as in Figures 2 and 3 except that these cells have been irradiated with 15,000 r and incubated for two and one-half hours. Many of the nuclei are bilobed, and one nucleus has a large vacuole. Although most of the cells appear morphologically intact, the cells do not have any oscillatory movement in the projected film and the cells in a few hours underwent autolytic changes. Nearly all of these cells are presumably dead by delayed fixation.

sions derived from a lymph node usually contained a small percentage of cells which were dead by fixation as a result of the trauma during the preparation of the suspension. As these fixed cells were somewhat difficult to differentiate from viable cells, it was advantageous to incubate the slide preparations of lymph nodes for two hours before making a viable cell count.

Other authors have studied cell death by phase microscopy. Bessis [2] described cell death associated with karyolysis, pyknosis, cytoplasmic fragmentation, cytoplasmic vacuolation, and other changes. Zollinger [18] described death associated with a brilliant, intermediate, or hazy nucleus.

Counts of Viable Lymphocytes.—Counts were made of the number of viable lymphocytes in a long narrow strip almost completely across the drop of suspension. A disk with a rectangular field of view subdivided into four smaller rectangles was inserted in one ocular. The slide was slowly moved by means of the mechanical stage, and the viable lymphocytes crossing a line in the ocular field were counted. This line measured 0.0215 mm. in apparent length with the × 45 objective. The mechanical stage was equipped with a micrometer screw which moved the stage 0.5 mm. on one rotation of the screw. A rectangular strip 0.0215×0.5 mm. was considered as one area for counting. Usually counts were made of the cells in 20 consecutive areas, i. e., in a strip 0.0215×10 mm. The present method of counting cells is somewhat similar to that described by Utermöhl [16] for counting plankton.

Microscopic surveys of the slides indicated, in general, satisfactory distribution of the cells. At the edge of the drop the cells tended to be slightly concentrated, especially if the petrolatum ring was small for the size of the drop. To minimize the effect of this irregularity in distribution, the count was started about 1 mm. from the petrolatum.

Tests were made on the uniformity of the distribution and on the accuracy and reproducibility of the counts. In a typical experiment with cells from a patient with chronic lymphocytic leukemia, the distribution was checked by counting the number of cells in each of 20 consecutive areas in three slides (Table). In Slides 1 and 2 the distributions seemed to be satisfactory, although they were not analyzed statistically. In Slide 3 the counts were somewhat irregular and variable and the distribution curve had two maxima. In three additional slides the total counts for the 20 areas were obtained. The average number of cells per area was 18.4. The average deviation of an individual count was 7.4%, and the maximum deviation was 20% for Slide 3.

Variability in Counts of the Six Slides and in Counts per Area in Slides 1, 2, and 3 *

Slide No.	Average No/Area	No. of Areas with Counts of 4-9	10-15	16-21	22-27	28-33	34-
1	18.0	0	8	9	2	0	1
2	17.7	2	7	4	4	3	0
3	22.9	2	0	9	2	5	2
4	17.2						
5	17.4						
6	17.1						
Average	18.4						

*Number of viable lymphocytes in 6 slides prepared from the blood of a patient with chronic lymphocytic leukemia.

From the data that the slides had an average of 18.4 viable lymphocytes per area and that the thickness of the metal separators and of the drop was 0.88 mm., it was calculated that the suspension contained 1900 viable lymphocytes per cubic millimeter. This determination compared favorably with the original count of 1600 viable cells by the method of unstained cell counts. This and other studies demonstrated the feasibility of using the proposed methods.

The slide preparations were counted daily to determine the number of viable lymphocytes. The percentage of surviving cells was calculated, based on the original count as 100%. The results were graphed on semilogarithmic paper. The results of a typical experiment are presented in Figure 5, which shows the percentage of surviving cells in a suspension from a lymph node of a patient with chronic lymphocytic leukemia. Varying amounts of mechlorethamine hydrochloride had been added to the suspension to give final dilutions of 0.3μg. to 30μg. of mechlorethamine hydrochloride per milliliter of mixtures. The 10% survival time for normal lymphocytes was 6.8 days.

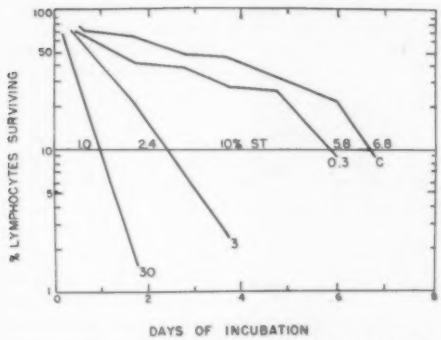

Fig. 5.—Effect of mechlorethamine hydrochloride (HN_2), on the survival curves of lymphocytes obtained from a lymph node of a patient with chronic lymphocytic leukemia. The slide preparations contained 30μg., 3μg., 0.3μg., and 0μg. of mechlorethamine hydrochloride per milliliter. The corresponding 10% survival times were 1.0, 2.4, 5.8, and 6.8 days.

The cells treated with 3μg. of mechlorethamine hydrochloride per milliliter of suspension had a 10% survival time of 2.4 days. This dosage of mechlorethamine hydrochloride caused an appreciable decrease in the survival time of the lymphocytes.

Prolonged microscopic observations and time-lapse cinemicrographic studies failed to show any mitotic divisions in the slide preparations. The original cells sometimes underwent both an increase in size and change in morphology, but they did not divide or increase in number. The slide preparations showed cell survival rather than cell culture.

Comment

The objective of the present work was to develop a method to test the sensitivity of the lymphocytes from blood or lymph nodes of the individual patient with chronic lymphocytic leukemia to the therapeutic agent proposed for the patient. The method described in this paper permits such tests and also permits a comparison of the patient's cells with normal lymphocytes with respect to sensitivity to the therapeutic agent. The method is being used in this laboratory to measure the sensitivity of leukemic and normal lymphocytes to x-rays and radiomimetic agents. A report has been published [14] on some of the early findings.

In addition to the quantitative measures of sensitivity, the slide preparations permitted qualitative morphologic observations on the behavior of treated and untreated, leukemic and normal, lymphocytes. Direct visual microscopic and time-lapse cinemicrographic studies were made to determine the appearance and the behavior of the cell before, during, and after cell death. The cinemicrographic films were also useful in teaching the recognition of viable cells and increased the accuracy of the viable cell counts.

The terms "viable" and "dead" when applied to cells have different connotations to different biologists. The bacteriologists consider a bacterium as dead when it has lost its ability to form a colony on an agar plate or to produce visible growth in broth.[11] Some cytologists have adopted the same definition for mammalian cells. For example, Puck and Marcus [10] defined a viable tumor cell as one that can produce a macroscopic colony in tissue culture. Similarly, Hoskins et al.[5] considered a tumor cell as viable if it could produce a visible tumor in a completely susceptible host. These investigators used the ability of a cell to multiply indefinitely as the only final criterion on viability.

Are these technical definitions of viability and death satisfactory? Rahn [11] pointed out that the ability to reproduce is an unusual criterion of death, but he defended the definition as a matter of necessity and convenience in testing for the death of bacteria. The definition has to be limited to cells, since ability to reproduce is not used as a criterion of viability of the whole animal. Even at the cellular level, the definition leads to difficulties and incongruous conclusions. For example, the neurones of the brain cannot multiply and have to be considered as dead, according to the definition. Bacteriologists were surprised when Kelner [7] found that bacteria considered dead as a result of exposure to ultraviolet irradiation could regain their viability by exposure

to visible light. Finally, it seems strange to read that a "killed tumor cell will still multiply several times, corresponding to four or five divisions." The question arises as to how many successive mitoses are needed to prove a cell alive. The definition of viability as synonymous with limitless reproducibility seems unusual, inappropriate, and limited in scope.

Tullis [15] could not use Puck and Marcus's definition of viability for the nondividing leukocyte. He implied that the loss of phagocytosis, motility, or any other function of a cell is a loss of viability. He criticized the use of resistance to staining with eosin as a test of viability, since this faculty is one of the last to be lost by the cell. Tullis seems to equate viability with the normal, physiologic state of a cell; i. e., viability becomes synonymous with vitality or health.

A more satisfactory approach to definitions was taken by Cameron,[3] who differentiated between death and injury. A cell—or an animal—may be considered viable even if injury, disease, differentiation, or aging caused the loss of such properties as motility or the capacity to multiply. Although no final definition can be given of viability or of life, it would be reasonable to define a cell as dead only if it shows signs of severe irreversible injury. This definition seems to be in accord with the views of Cameron [3] and Müller [9] and could be applied in principle to a tumor cell, to a leukocyte, and to the intact animal. The definition may, however, be difficult to apply in specific instances. The difficulty can be circumvented by labeling a cell with a neutral term as altered, defective, inactivated, or injured if there is any question whether an observed change means cell death.

In the present study the counting of the "viable" lymphocytes presented two distinct problems—one a question of semantics and the other a question of observation. In this work it seemed reasonable to consider the violent morphological changes of intranuclear vacuolation and the subtle changes called delayed fixation as signs of loss of viability. To eliminate the question of semantics it might be preferable to use a more objective phrase, such as "morphologically intact and unaltered" lymphocyte. This descriptive but awkward phrase stresses observation rather than arbitrary definitions. For example, irradiation with 1000 r produced changes in 11.2 hours, so that very few cells could be recognized as intact lymphocytes (Fig. 3), while the nonirradiated lymphocytes remained morphologically unaltered after 21.9 hours of incubation (Fig. 2). This finding can be retested in this and other laboratories, and the validity of this observation does not depend on terminology.

It may be concluded that the slide method proposed in this paper permits the qualitative study of the morphologic changes produced by a reagent to normal and leukemic lymphocytes. It also permits the quantitative determination of the capacity of the reagent to produce morphologic changes in the cells.

Summary

This paper presents a qualitative and quantitative method for studying the morphologic effects of reagents to human lymphocytes obtained from normal persons and from patients with chronic lymphocytic leukemia. Lymphocytes from normal and leukemic blood were isolated by means of differential sedimentation and centrifugation and suspended in 50% normal human serum. A small number of granulocytes and other cells were also present in the suspensions, especially those derived from normal blood. Similar cellular suspensions were prepared from human lymph nodes removed for biopsy. The suspension with or without the reagent were put up in slide preparations which contained 0.2 ml. of suspension in the form of a thick drop. Morphologic changes induced by the reagents were studied by direct phase microscopy and by time-lapse cinemicrography. With most reagents, viable, i. e., morphologically intact and unaltered, lymphocytes could be differentiated from dead lymphocytes and from other cells

by phase microscopy. The viable lymphocytes were counted daily in a measured area across the central part of the drop. In one experiment, for example, leukemic lymphocytes from a lymph node had a 10% survival time of 6.8 days. The addition of mechlorethamine hydrochloride to a concentration of 3μg. per milliliter of suspension reduced the 10% survival time to 2.4 days. The slide method thus provides qualitative and quantitative data on the cytotoxicity of reagents to normal and leukemic lymphocytes.

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Cystic Teratomas of the Ovary

A Clinical and Pathological Analysis of Two Hundred Sixty-Eight Tumors

RAUL A. MARCIAL-ROJAS, M.D., and RUBEN MEDINA, M.D., San Juan, P. R.

Although the morphology of the ovarian dermoid cysts is grossly and microscopically well established, for most clinicians a dermoid cyst still implies an ovarian cystic tumor containing large amounts of buttery pultaceous material admixed with numerous hairs and occasionally presenting portions of bone or teeth. Careful histologic analysis from different areas of these tumors frequently discloses numerous other tissues derived from all primitive germ layers. This variety of tissues encountered very much obscures the boundaries between these tumors and those of the teratoma group.

Only a few well-documented series of dermoid cysts of the ovary have been published. The two largest series are those of Koucky,[1] in 1925, and of Blackwell et al.,[2] in 1946. Both reports are from the Mayo Clinic; the former is a complete clinical and pathologic analysis of 100 dermoids, and the latter, a clinical analysis of 225 tumors, with pathologic studies of 100 of them. Blackwell et al. make no mention of the work of Koucky, so one is unable to determine if there is some overlapping in the cases analyzed Most of the other papers published on the subject have been for the most part based on the study of single cases or small series of cases.[3-6]

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The wealth of material at the Department of Pathology of the University of Puerto Rico School of Medicine, School of Tropical Medicine, coupled with our feeling that a review of a large series of dermoids might provide some valuable information regarding their histopathology, clinical manifestations, and incidence of malignant degeneration, motivated us to undertake the present study.

Materials and Methods

From 1940 to 1953, inclusive, 48,316 surgical specimens were grossly and microscopically examined at the Department of Pathology of the University of Puerto Rico School of Medicine, of which 32,000 (68%) belonged to female patients. Of the latter, 2796 (8.5%) contained ovarian tissue. Among these, 417 primary tumors of the ovary were encountered, from which 268 dermoid cysts belonging to 229 patients were collected. The clinical history of all the patients was analyzed, and at least two sections of each tumor were studied microscopically. These were routinely stained with hematoxylin and eosin. Special stains and methods were used to demonstrate some components in the tumors, such as mucus, bone, etc.

Incidence

Dermoid cysts make up a considerable proportion of the neoplasms of the ovary. Their incidence is usually stated to be from 5% to 10% of all ovarian tumors[7]; however, there are several large series in which their incidence varies between 5% and 34%.[8] The incidence in this series was 53.5%, which is the highest recorded in the literature. Our series is also the largest one so far reported.

The tumors show no predilection for either ovary. In 17% of the 229 cases the tumor was bilateral. Quinland and St. Hill[9] state that their bilaterality varies from

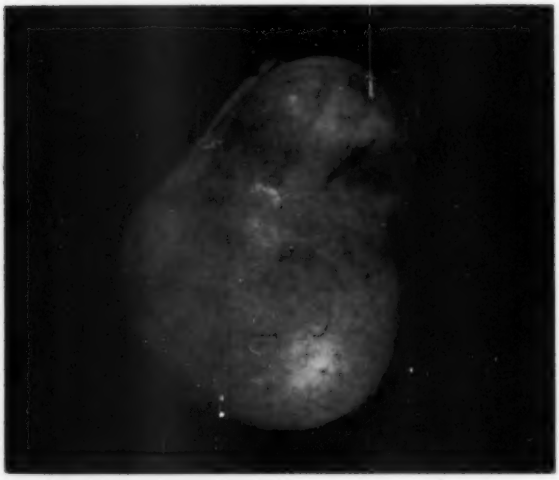

Fig. 1.—Usual appearance of a dermoid cyst.

10%-40%. They were encountered in patients of all ages. The youngest patient in the group was 3 and the oldest, 77 years of age. Of the tumors in this series, 86.4% were encountered in patients between the ages of 11 and 50 years. Therefore, the greatest incidence was during the active reproductive life of the patient. It is the opinion of most authors that those tumors found after the menopause had their origin before the termination of ovarian activity.

Gross Appearance

The shape of most of these tumors was globular, but not infrequently they were ovoid and even pyriform. The wall was parchment-like, smooth, and glistening and had prominent superficial blood vessels. The color in the great majority was white, often with yellowish areas imparted through the wall by the contents. When there was torsion of the pedicle with infarction, the wall was red and hemorrhagic.

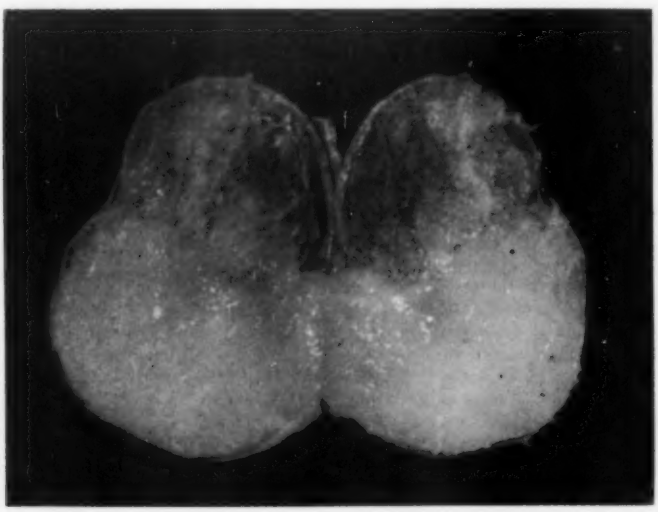

Fig. 2.—Cut surface of dermoid cyst, showing hair and pultaceous material.

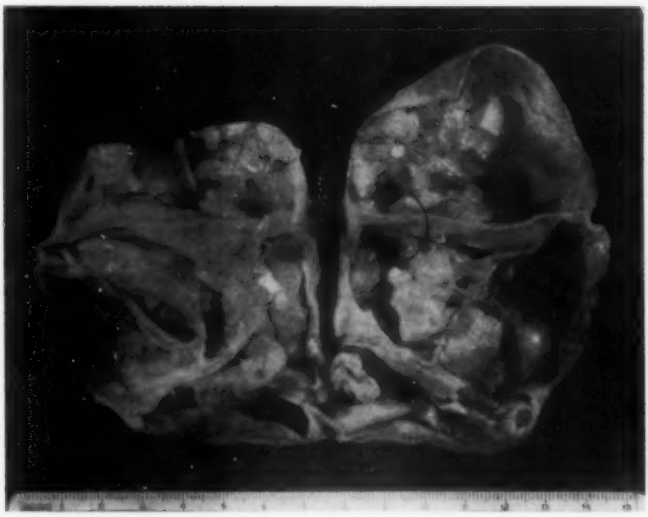

Fig. 3.—Dermoid, showing two well-formed molars.

The tumors were soft and cystic immediately after excision, but later on their contents became doughy and semisolid at room temperature. The consistency varied according to the presence of bone, cartilage, teeth, or calcification of the wall.

The contents were oily, pultaceous, and admixed with masses of hair and frequently teeth and bone. At a point in the inner surface there usually protruded a sessile mass covered with thick skin and on section disclosing beneath, well-developed adipose tissue with occasional bone or teeth. The remainder of the inner lining was usually smooth, as in serous or pseudomucinous cytomas, but occasionally granular, shaggy, and ulcerated. Most of the latter had marked thickening of the wall by fibrosis and hyalinization, with areas of calcification.

The color of the hair bore no relation to the age or to the color of the patient's hair.

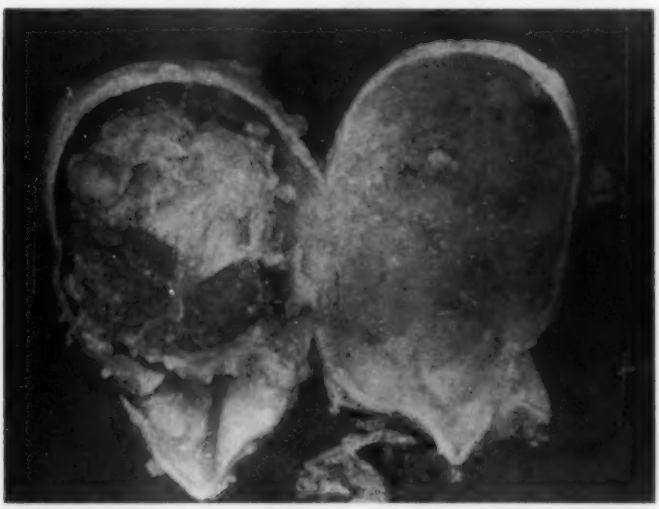

Fig. 4.—Ulceration and calcification of wall of dermoid cyst.

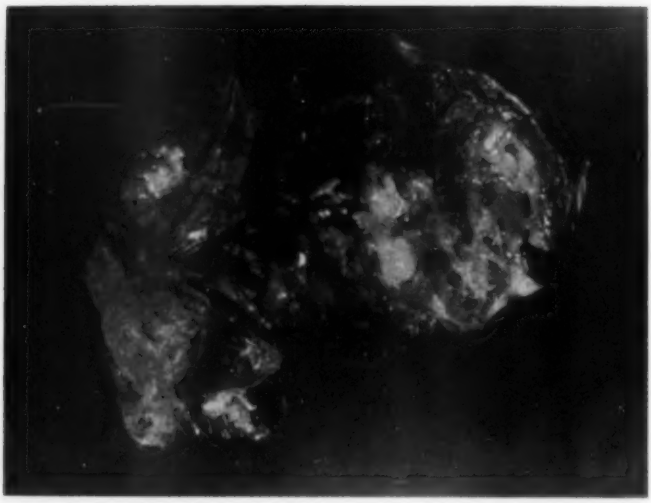

Fig. 5.—Dermoid cyst with hemorrhagic infarction due to torsion.

In this series teeth were encountered in 26% of the cases. They usually resemble adult molars, but occasionally, incisors. Most of these were found embedded in the wall of a rudimentary bony structure closely simulating mandible or maxilla and only rarely were lying free in the cavity of the cyst.

The size of the tumors varied from 9 mm. (the smallest) to 29 cm. (the largest) in transverse diameter. The average size was 7.9 cm., and they seldom attained a size larger than 17 cm. No definite relationship could be established between the size of the tumor and the age of the patient.

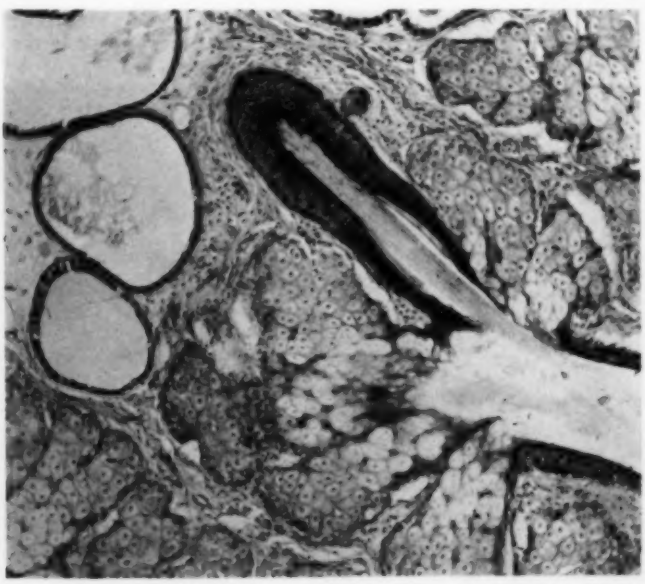

Fig. 6.—Section from mammilla, showing hair follicles and sebaceous and apocrine glands; × 80.

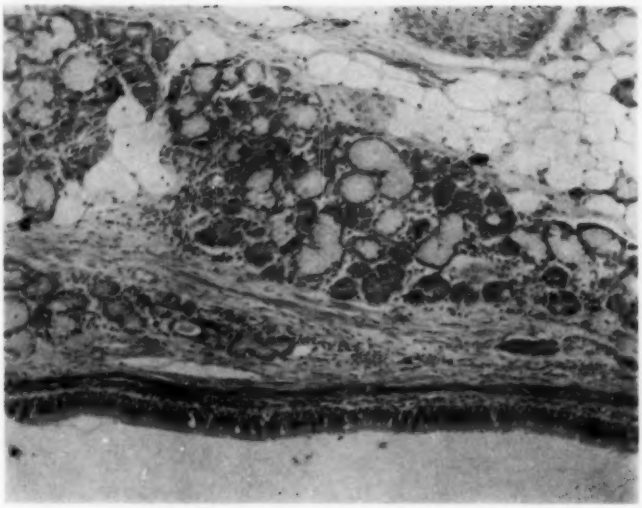

Fig. 7. — Respiratory epithelium with submucosal glands forming the wall of a bronchus; × 80.

Microscopic Examination

At least two sections of each tumor were examined histologically. As a rule the microscopic study revealed numerous structures and tissues by far greater in number than expected from the gross appearance. The three germ layers were represented in the great majority of the tumors.

Ectodermal structures were universally encountered, as 100% of the tumors showed areas of stratified squamous epithelium lining the cyst. This was difficult to recognize in those tumors with hemorrhagic infarction or severe inflammation secondary to ulceration, but when several sections were taken at random it was always demonstrated.

Sebaceous glands were abundantly present in 98% of the tumors. They are undoubtedly responsible for the sebaceous contents of these cysts. In 96% hair follicles were seen. Sweat glands were present in

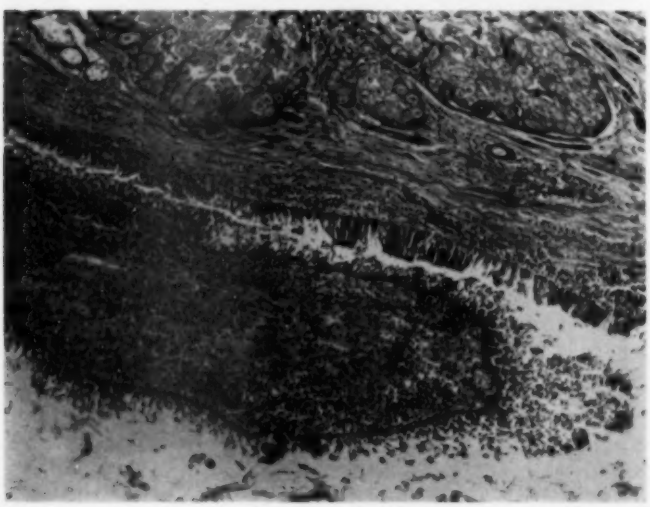

Fig. 8. — Respiratory epithelium covering an adenoid-like structure; reduced about 10% from mag. × 80.

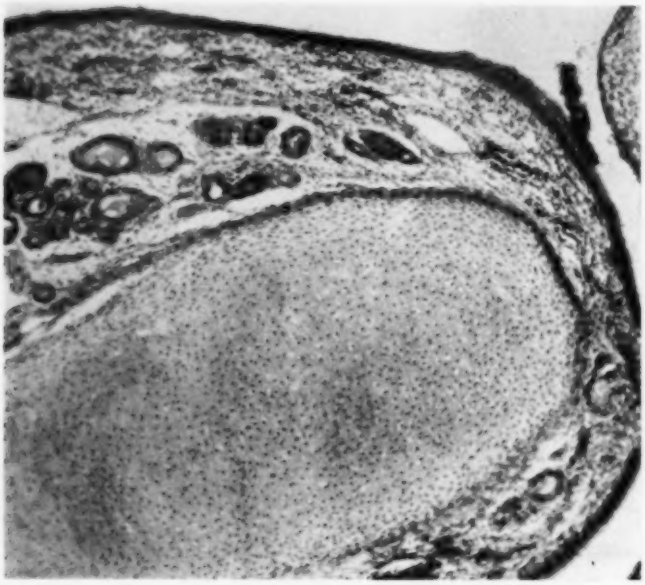

Fig. 9.—Structure resembling epiglottis; × 80.

75%, and apocrine ones, in 52% of these series.

Structures pertaining to the central nervous system were frequently encountered. Brain tissue was evident in 41% of the group. It is of interest to mention that the presence of cerebral tissue could hardly be predicted in gross examination. Many times the lining of the cyst was made of a narrow band of cerebral cortex. Ependymal epithelium was encountered in 19% of the tumors and in 6% of them

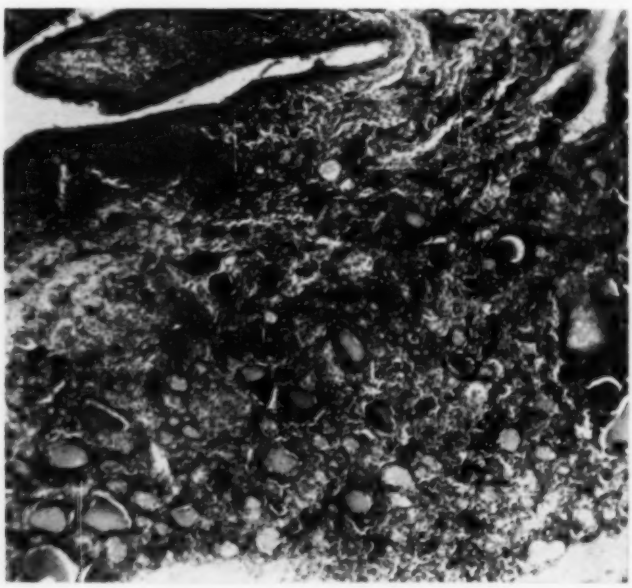

Fig. 10.—Thyroid tissue in dermoid; × 80.

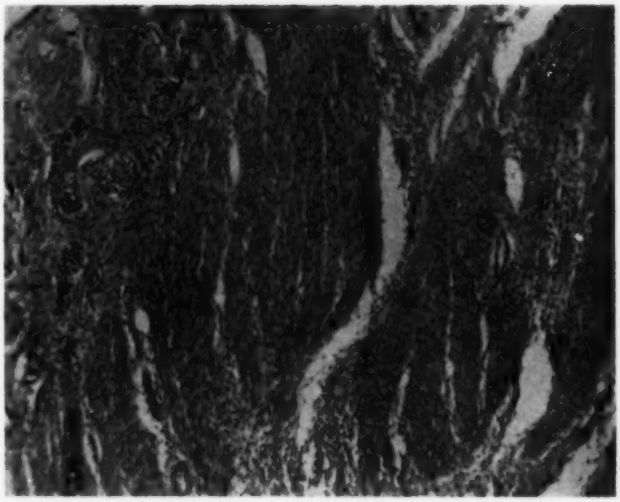

Fig. 11.—Smooth muscle forming a well-delimited mass appearing like a leiomyoma; reduced about 10% from mag. × 80.

well-developed choroid plexus. Cerebellar tissue was found in 2%, and structures resembling retina, in 1%.

Thirty-eight per cent of the group had peripheral nerve fibers, and twenty-two per cent, ganglionated cells.

Mesodermal derivatives were frequent and occurred in 92% of the tumors. Smooth muscle was usually found in the form of bundles near the skin appendages, mostly hair follicles, or as a support to gastrointestinal or tracheobronchial epithelium. It was found as such in 92% of the cysts. One of the tumors had a localized mass of smooth muscle in the wall, practically fulfilling the criteria of a leiomyoma. Striated muscle was never seen.

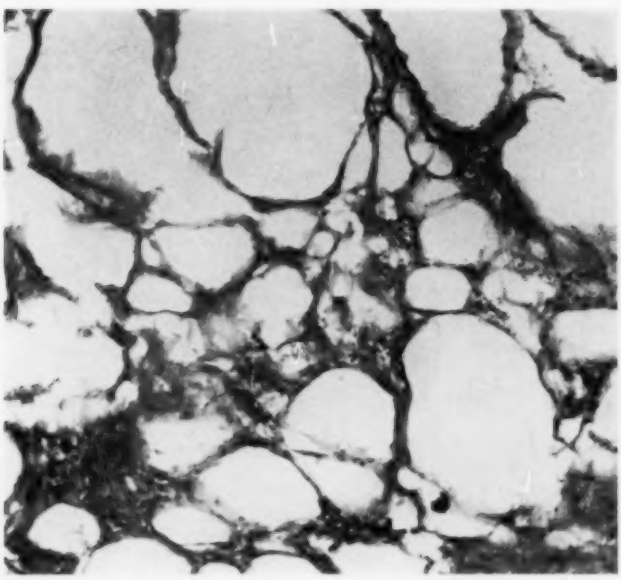

Fig. 12.—Sieve-like areas almost pathognomonic of "dermoid cyst peritonitis"; reduced about 5% from mag. × 80.

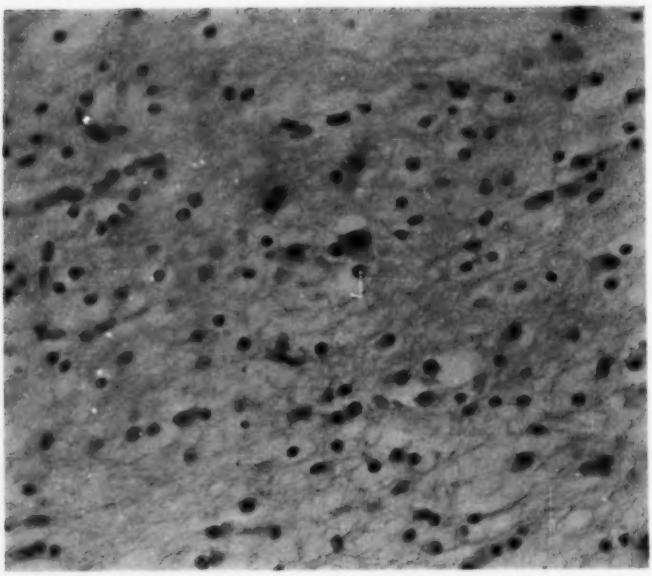

Fig. 13.—Cerebral tissue; reduced about 5% from mag. × 360.

Bone was present in 35%, and slightly more than one-third of these contained well-formed bone marrow. Cartilage was evident in 22%.

Structures derived from endoderm were encountered in 72% of the cases. Serous mucous glands appeared in 11%, and gastrointestinal epithelium, in 13% of the

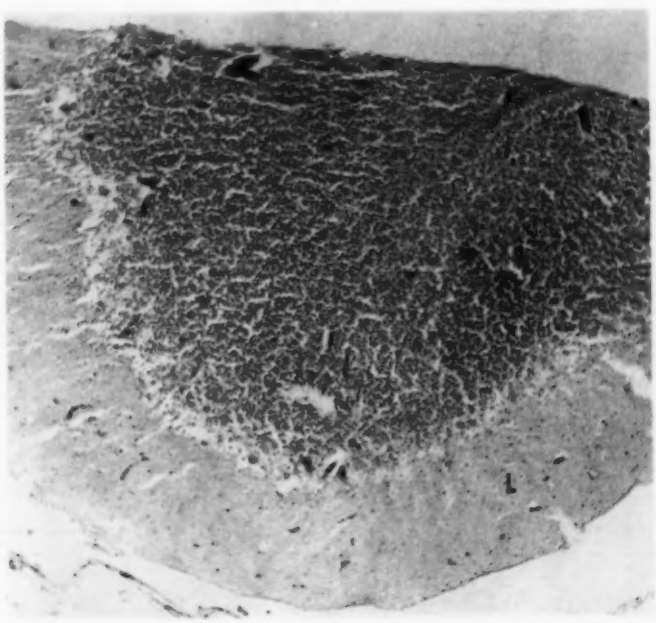

Fig. 14.—Cerebellar tissue; reduced about 5% from mag. × 80.

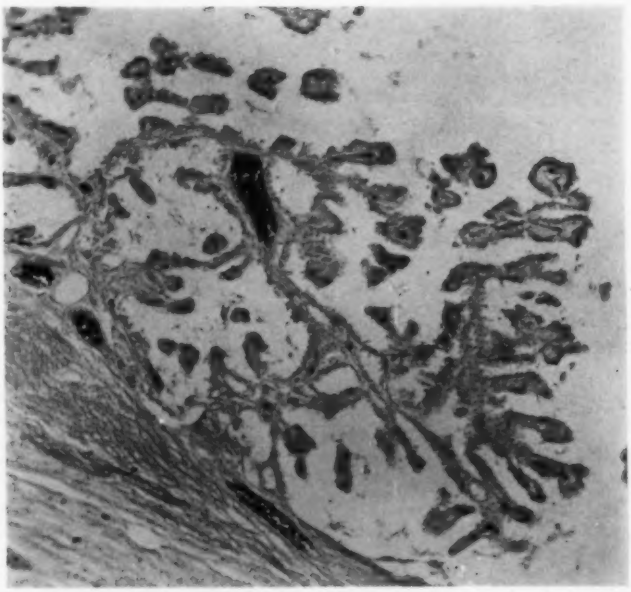

Fig. 15.—Choroid plexus; × 80.

group. Respiratory epithelium was found in 48% and frequently associated with cartilage, smooth muscle, and bronchial glands in an attempt to form a bronchus.

In 7% of the lesions thyroid tissue was evident. In some of them it was found in moderate abundance, but never in quantities large enough to detect it in the gross examination.

Ulceration of the lining of the cyst was rather frequently encountered. Forty-three per cent of these lesions showed partial or

Fig. 16.—Ependymal-lined cavity; × 360.

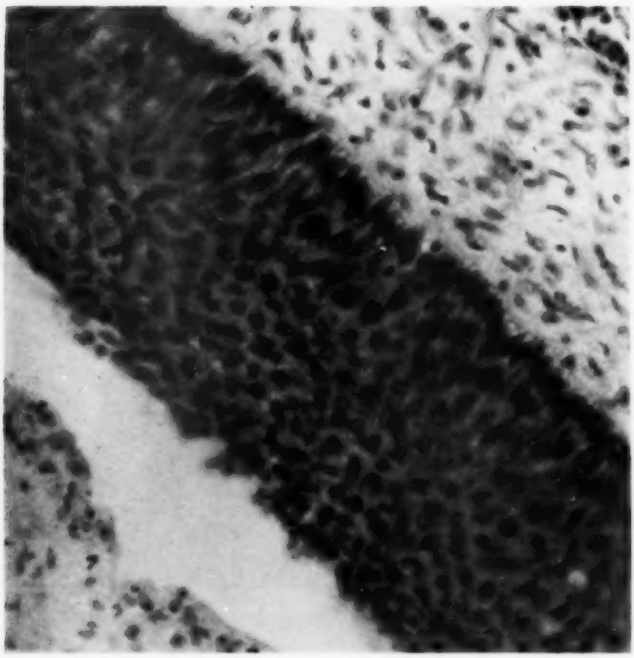

Fig. 17.—Intraepithelial carcinoma in dermoid cyst; × 360.

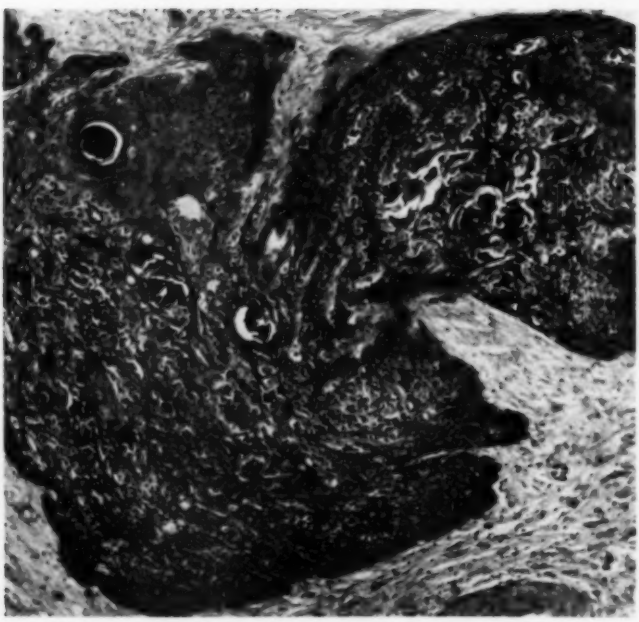

Fig. 18.—Invasive epidermoid carcinoma arising in dermoid; × 80.

complete ulceration with chronic inflammation. The latter is characterized by lymphocytes, numerous foreign-body giant cells, foamy macrophages, and areas of granulation tissue. Most of these also revealed many sieve-like areas in the wall formed by flattened giant cells. In some the process extended into the adjacent peritoneum and peritoneal fat, producing a granulomatous reaction simulating tuberculosis.[10] In two cases such diagnosis was erroneously made histologically. It seems to us that this granulomatous type of reaction described is almost pathognomonic of dermoid cysts.

Analysis of Presenting Complaint

A tedious search was made to analyze the records of all patients concerning their presenting complaint. This was satisfactorily accomplished. Additional information regarding menstrual history, parity, abnormalities of menstruation, and associated pelvic pathologic conditions was so fragmentary that could not be adequately evaluated.

The presenting symptoms in our series can be readily summarized. It can also be safely stated that the symptoms associated with dermoid cysts of the ovary have no diagnostic differential value.

Of the 229 patients with dermoid cysts of the ovary, 190 had dull pain in the lower abdomen, with or without a sensation of fullness and pressure symptoms, mostly manifested by frequency in urination. In all these patients a mass was palpable.

The ovarian dermoids of 13 patients were encountered incidentally at exploratory laparotomy. These were usually small or associated with leiomyomata of the uterus.

Twenty-six patients were admitted with a diagnosis of an acute abdominal catastrophe. In all of them a twisted dermoid cyst with varying degrees of hemorrhagic infarction was encountered.

Complications

The most frequently encountered complication in our series was that of torsion and hemorrhagic infarction. It occurred in 10% of the cases and was manifested clinically as an acute abdominal emergency. This surgical problem was easily recognizable in all the cases, but the exact type of ovarian pathology could not be diagnosed from the symptomatology. No mortality was encountered for this complication in our series, and all the patients recovered. The incidence as to age of torsion and hemorrhage is essentially the same as for the general group of dermoid cysts.

Malignant degeneration is the most dreaded complication of cystic ovarian teratomas. There were six instances in our series, for an incidence of 2.2%. Four of the cases were truly invasive epidermoid carcinomas, one was an intraepithelial cancer, and the other was a malignant melanoma. The latter represents the third authenticated primary ovarian melanoma reported in the literature.[11]

A rather infrequent complication, only encountered in three cases in our series, is one which should be labeled as "dermoid cyst peritonitis." It represents a peculiar granulomatous inflammatory process provoked by the contents of the cyst. Owing to the ulceration of the inner lining and rarely to rupture of the wall of the cyst, there is seepage of the pultaceous contents of the cyst into the wall and adjacent tistues. This material produces a granulomatous type of inflammatory process which sometimes has been confused with tuberculous peritonitis or it may even mimic metastatic carcinoma.[12] In two of our cases it was originally diagnosed microscopically as tuberculous peritonitis.

Comment

The present clinical and pathologic analysis of 268 dermoid cysts of the ovary has provided us with several interesting facts about their incidence, histopathologic structure, and complications.

The incidence of these tumors varied in previous reports from 5% to 34% of all ovarian neoplasms. Our series revealed a

much higher incidence, 53.5%. We should not consider this an absolute figure, but merely one of temporary geographical consequences. The population of Puerto Rico is a very young one when compared with that of the United States, so we lack in these series our share of malignant tumors of the ovary, which are by far more frequent in the sixth and seventh decades. With increase of average life span, from 40 years in 1930 to 68 years in 1956, our population in the near future will attain the same average age as that of the United States, and in all probability the incidence of the different types of ovarian neoplasms will be the same in both countries.

It is clearly evident from this analysis that dermoid cysts of the ovary exhibit by far a much greater multiplicity of derivatives of the different primitive germ layers than implied by their name. We are sure that the incidence of endodermal and mesodermal derivatives in these tumors will closely approximate 100% if numerous sections are taken from different areas of the tumor. Because of this, dermoid cysts should be referred to as teratoid cysts or cystic teratomas to differentiate them from the solid ovarian teratomas.

The most frequent complication of cystic teratomas in this group was that of torsion and hemorrhagic infarction. Malignant degeneration was encountered in 2.2% of the cases. Counsellor and Wellbrock,[14] in 1934, found 7 cases of epidermoid carcinoma out of a total of 408 dermoids surgically removed at the Mayo Clinic, or 1.7%. This figure corresponds with that of John Miller,[15] in a collective review of 1268 dermoid cysts. One of us (R. A. M-R.) has had the opportunity to see and study eight instances of invasive epidermoid carcinomas arising in dermoid cysts of the ovary. These are going to be the subject of a separate communication,[13] but we want to emphasize here the importance of careful study of the wall of all teratoid cysts. We have occasionally seen small areas of thickening resembling compressed fibrotic ovarian tissue which microscopically has proven to be epidermoid cancer. Also areas of hyalinization with ulceration may disclose invasive squamous-cell carcinoma. Sections should be obtained from all these areas of thickening.

Other malignant tumors may develop in teratoid cysts. There is an authenticated primary malignant melanoma in this group. We have not seen carcinoid tumors, but a group of them have been reported. A primary *leiomyosarcoma* arising in a dermoid in a case of bilateral dermoid cysts is soon to be reported by one of us.[18]

Summary and Conclusions

A clinical and pathologic analysis of 268 dermoid cysts of the ovary from 229 patients is attempted.

The high incidence of these tumors in our series, 53.5%, can be explained on the basis of the very young population existent in Puerto Rico at the present time.

In 17% of the cases the tumor was bilateral.

Of the tumors, 86.4% were encountered in patients between the ages of eleven and fifty years.

The tumors did not show any predilection for either ovary.

The average size was 7.9 cm. in diameter.

Ectodermal derivatives were universally encountered. Endodermal derivatives were seen in 72%, and mesodermal ones, in 92% of the cases.

If more than two sections are taken from the cysts, in all probability the incidence of both mesodermal and endodermal derivatives will closely approximate 100%.

Because of the obvious composition of most of these tumors from the three primitive germ layers, they should be labeled as "teratoid cysts," or better *"cystic teratomas."*

Malignant degeneration, usually in the form of epidermoid cancer, was encountered in 2.2% of our cases.

It is apparent that the symptomatology of most of these tumors is far from being diagnostic of the condition.

Removal of the cysts is the treatment recommended, especially in view of a 10% incidence of torsion and hemorrhagic infarction and 2.2% incidence of the more dreaded and usually fatal malignant degeneration of these cysts.

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Intramural Fibroma of the Myocardium

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Introduction

Although primary neoplasms of the heart are rare, the increasing number of reported cases is apparently commensurate with the increasing interest in congenital heart disease. One of these rare types of primary tumor of the heart is the intramural fibroma. Several have been reported in the American literature, and a few described in the European literature.

One of the earliest recorded tumors of the heart was described by Colombo, in 1559, cited by Mahaim [1]; this tumor was both fibrous and polypoid. Clerici and Teuscher, cited by Stout,[2] reviewing the subject, found cardiac tumors predominantly benign and true fibromas infrequent. In a more recent review, Prichard [3] separated intramural fibromas of the heart from polypoid fibromas and fibromatous lesions of the valves—the distinction being made because the latter occasionally occur as a result of infectious valvular disorders or attached thrombus material. The literature reveals that the majority of intramural fibromas have been found in children (Table), that death occurs suddenly and unexpectedly, and that the diagnosis is made only at necropsy.

The purpose of this paper is to add a case to those already reported in the literature and to summarize current beliefs concerning this unusual entity.

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Park Clinic (Dr. Krueger); Pathologist, St. Joseph's Hospital (Dr. Knoll).

Report of Case

A white girl of 4 years, while watching television with other members of the family, suddenly collapsed. During the previous 48 hours she had seemed well except for symptoms of a mild respiratory infection. The child was dead on arrival at the hospital emergency room several minutes after her collapse at home.

In retrospect, a review of her history revealed that her health had been considered good. She had been seen two years previously by a physician, whose findings on physical examination and laboratory studies had been within normal range except for a slight anemia, which was not treated.

Gross Autopsy Findings

Autopsy disclosed a well-developed, well-nourished girl 100 cm. in length and 40 lb. in weight. Although the most significant pathology was present in the heart, the following general findings are of interest: The skin was moderately cyanotic, and the teeth, somewhat carious. The brain was edematous, weighing 1575 gm. The lungs were congested and edematous, the com-

Myocardial Fibromas Reported in the Literature

Author	Year	Age of Patient	Sex	Location of Tumor
Luschka (Mahaim [1])	1855	6 yr.	M	Left ventricle
Wacstaffe (Mahaim [1])	1871	3yr.	F	Interventricular septum
Lefas (Mahaim [1])	1900	----	--	----
Biemann (Mahaim [1])	1914	----	--	----
Monkeburg [8]	1924	Infant	--	Left ventricle
Teuscher (Mahaim [1])	1927	2½ yr.	F	Left ventricle
Symeonidis & Linzbach [4]	1938	15 mo.	M	Left ventricle
Kulka [10]	1949	8 mo.	F	Left ventricle
Bigelow et al. [7]	1954	3 days	F	Left ventricle
McCue et al. [6]	1955	4 yr.	F	Interventricular septum & left ventricle
James & Stanfield [11]	1955	4 yr.	F	Left ventricle
Conlon [12]	1956	7 mo.	M	Left ventricle

Fig. 1.—Bisected left ventricular wall, showing tumor.

bined weight being 310 gm. Each pleural space contained 3 cc. of serous fluid. Two small anomalous lobes were united to the right lobe of the liver, the entire organ weighing 650 gm. Marked lymphoid hyperplasia was observed in the wall of the cecum.

The pericardial sac contained 15 cc. of amber fluid. The enlarged heart, bulging in its left lateroposterior aspect, presented an egg-shaped contour and weighed 114 gm. Bisection of the left ventricular wall disclosed a solitary, gray-white, oblong tumor mass involving almost the entire structure (Fig. 1). The mass measured 7 cm. in the direction of the cardiac axis and 2.6 by 2.5 cm. in the opposite planes. The tumor caused a bulging contour which extended, in part, cephalad to the atrioventricular groove. There was no definite capsule. In some regions, the growth was sharply demarcated, and in others, blended into cardiac

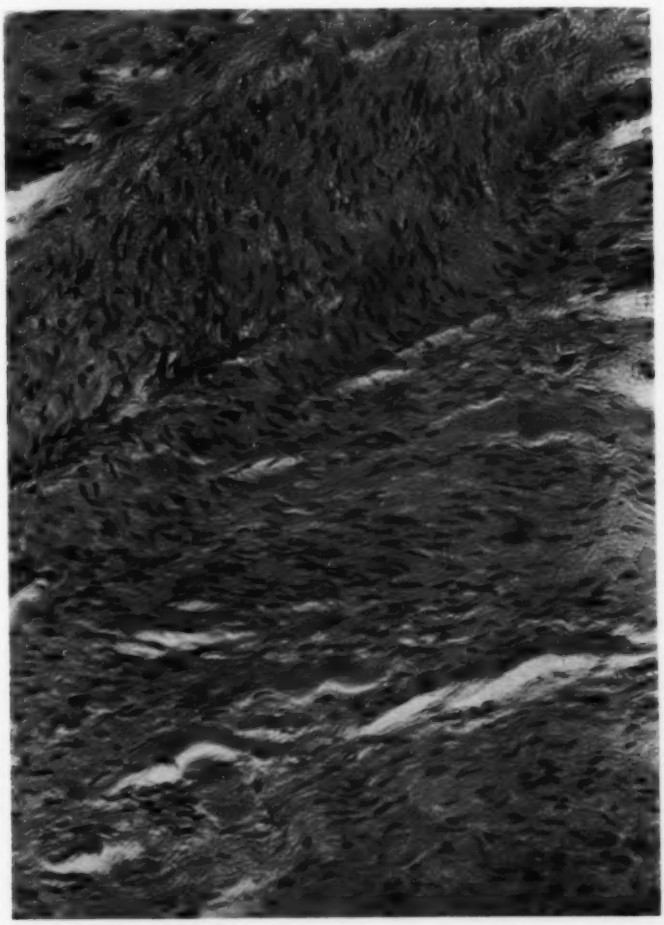

Fig. 2.—Fibrous tissue the main component of the tumor. Hematoxylin and eosin; reduced about 15% from mag. × 250.

muscle tissue. The tumor was firm, elastic in consistency, and definitely resistant to the knife, bulging above the cut surface with a faintly discernible whorled pattern. The heart valves and great vessels revealed no further pathologic changes.

Microscopic Autopsy Findings

Histologically, the tumor revealed large areas of fibroblasts with well-stained spindle-shaped nuclei and eosinophilic cytoplasm (Fig. 2). Collagen fibers intertwined with fibroblasts were also common. Within this fibrous tissue component were isolated muscle cells, as well as larger patches of myocardial tissue (Fig. 3). Such tissue was more frequent along the periphery of the tumor.

Special stains demonstrated cross striations of the pale tissue adjacent to these myocardial fibers and also elastic fibers. Occasional foci of calcification were noted in the fibrous structure. Blood vessels were few and frequently slit-like in outline.

At the periphery of the tumor, the adjacent myocardial fibers were often narrowed as if by pressure atrophy. Occasionally finger-like projections of the fibrous tumor extended into the adjacent myocardium, as described by Symeonidis and Linzbach.[4] The tumor was completely separate from the endocardial and epicardial surfaces.

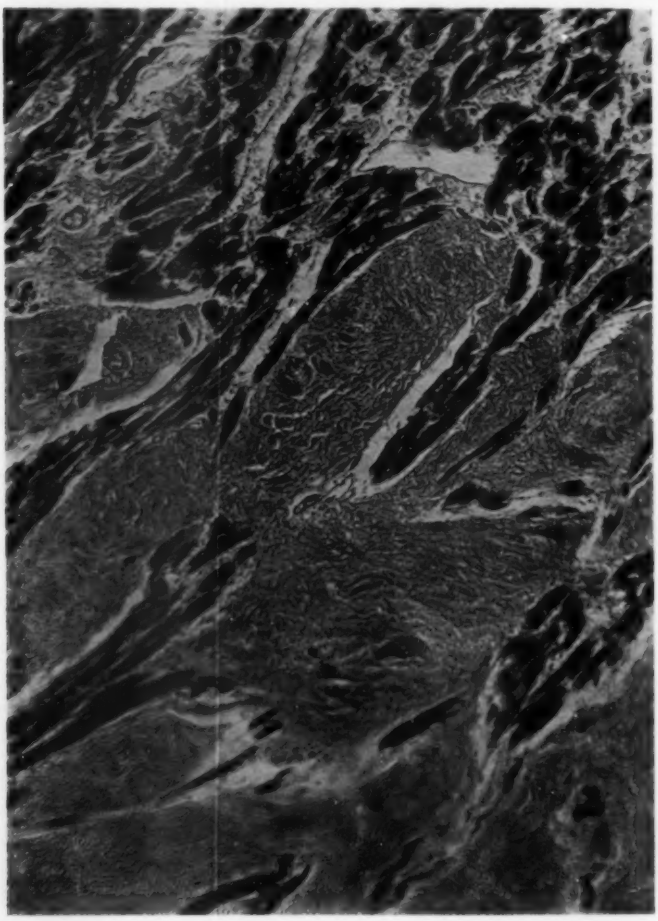

Fig. 3. — Relation of tumor to cardiac muscle fibers. Phosphotungstic acid-hematoxylin; reduced about 15% from mag. × 250.

The liver presented histologic evidence of chronic passive congestion; the lungs contained numerous pigment-laden macrophages and the eosinophilic coagulum of edema.

Anatomic Diagnosis

The anatomic diagnosis was primary intramural fibroma of the left ventricle with acute congestive heart failure.

Comment

The etiology of the so-called intramural fibroma of the myocardium is obscure. Stout [2] has pointed out that the heart is an organ of mesothelial derivation and that all of the undoubted tumors are composed of derivatives of this tissue. Both earlier and present writers have designated this lesion as a hamartoma of interstitial tissue of the ventricular wall.[3-5] Mahaim,[1] in his extensive monograph, proposed a classification of fibromas based mainly on gross appearance.

An explanation for the presence of isolated cardiac muscle fibers deep within the tumor mass is a subject for speculation. Fibromas of visceral organs may contain muscle fibers, according to Ewing.[6] Bigelow et al.[7] have considered the tumor to be an enlarging proliferating mass and the isolated muscle fibers within an inherent constituent of the neoplasm because of the primitive appearance of the latter. McCue and his colleagues [8] have more recently stated that this type of fibroma is one of the two kinds of congenital rhabdomyoma.

Summary

The myocardial fibroma is a tumor which apparently is of a congenital type, affecting the left ventricle or interventricular septum. It is almost invariably fatal in the first few years of life (Table).

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The Prognostic Implications of Vascular Invasion in Primary Carcinomas of the Lung

A Clinicopathologic Correlation of Two Hundred Twenty-Five Cases with One Hundred Per Cent Follow-Up

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The rapid and unique rise in frequency of primary pulmonary carcinoma over the past three decades and the apparent relationship to cigarette smoking [8,12-14,28-30,38,70,71,80] has made this neoplasm a subject of great interest and debate. The reality of this significant increase, early shown by Rosahn [65] and Simpson,[69] has been challenged by some workers [19,32,78] but sustained by numerous confirmatory studies.[25,56,62,65,66,69]

Despite advances in thoracic surgery associated with increasingly lower operative mortality, the end-results of therapy in carcinoma of the lung have been discouraging. The over-all five-year survival rates have ranged from 7% to 10% The five-year survival rates for operable cases have been, of course, considerably better but are still poor, ranging from 22% to 27.8%.[10,16,23,24,33,48,51,58,59] None the less, many long-term survivors have been reported.[24,32,55,63]

Pathologists and clinicians [24,36,64] alike have been forced to regard factors influencing survival as somewhat cryptic, for while some patients are apparently cured of their tumors after resection, other patients with tumors of the same histologic type, often less extensive, have gone on to death from carcinoma within the five-year period. With this in mind, the workers in this laboratory have searched for a common denominator which could be recognized from a study of the surgically excised lesions in order to offer the surgeon a more accurate prognosis than that afforded on the bases usually employed, namely, size, extent, and histologic type.

A review of the cases of lung carcinoma resected at the Hospital of the University of Pennsylvania confirms the observation by others that the undifferentiated carcinomas are uniformly associated with a poor prognosis. Beyond this, however, unlike the findings of Kirklin et al.,[35] histologic evaluation on the basis of morphologic type and degree of differentiation was not associated in our hands with a predictable and high correlation as to prognosis in any given case. Similarly, the size of the lesion did not always correlate with prognosis, a feature which has been emphasized by Coman and others.[11,63]

It has long been recognized that malignant neoplasms effect distant metastases via the blood stream in the form of tumor emboli, which have a particular predilection for the pulmonary vascular tree.[68] Such blood stream metastases have, as their usual genesis, vascular invasion at the site of the primary neoplasm. The diagnostic im-

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portance of vascular violation by cancer of the thyroid was pointed out by Graham [27] and Warren,[74] and its prognostic significance has been repeatedly emphasized by Warren and Meissner.[49,75,77] The prognostic impact of vascular invasion in carcinoma of the colon has been well documented by Fisher and Turnbull.[18] Extensive work on this subject is being carried out by Adamson.[2] Pool and Dunlop [61] have studied the effects of vascular invasion in cancer of the breast, colon, stomach, and rectum, as evidenced by tumor cells in the peripheral blood.

The first recorded evidence of vascular invasion by primary pulmonary carcinoma which we were able to find is in Wolf's study,[79] of 1895, in which a carcinoma of the main-stem bronchus invaded the right pulmonary vein and left atrium. Marcus,[42] in 1917, reported what he interpreted as neoplastic cells in the peripheral blood of a patient with proved bronchogenic carcinoma. Over the years, there have been many recorded instances of blood vessel invasion by primary pulmonary neoplasm.[3-5,7,9,20-22,45,60] Liebow's [49] classic demonstrations of pulmonary vascular channels, shunts, and communications, coupled with Batson's [6] work on spinovertebral veins, dramatized the potential cataclysm to the human organism following pulmonary vascular invasion by tumor. Despite these observations, no concerted emphasis has been placed upon the vascular invasion potential of primary lung cancer as a means of estimating prognosis. Therefore, we have instituted the study to be described.

Methods and Material

Two hundred twenty-six cases of resected primary pulmonary neoplasms operated upon at the Hospital of the University of Pennsylvania from November, 1939, until Dec. 31, 1955, were studied. Complete follow-up was obtained on all cases. The clinical and pathologic data of all patients were reviewed and incorporated on standard McBee "Keysort" punch cards. The pathologic data recorded included size and location of the tumor, histologic type, involvement of lymph nodes, freedom of lines of resection from tumor, and evidence of blood vessel invasion. In general, the sections studied included several levels of the lines of resection, representative blocks of tumor, and random sections of grossly uninvolved lung, plus multiple levels of resected lymph nodes. In only one case was the histologic material no longer available.

Following the suggestions of Warren [76] with regard to the total number of sections to be evaluated for vascular invasion in suspected cases three blocks of tumor which were recut and stained by the Verhoeff-Van Gieson technique or one of its of carcinoma of the thyroid, we routinely selected modifications.[72] Establishment of the histologic type was most frequently done on the basis of routine hematoxylin and eosin stains, but at times special stains proved helpful. Particularly valuable were the Alcian blue and periodic acid Schiff (P. A. S.),[58] the Alcian blue and Feulgen,[54] the Hotchkiss and McManus stain,[47] Mayer's mucicarmine,[48] Mallory's phosphotungstic acid hematoxylin stain,[41] and Gomori's trichrome stain.[30]

The classification of tumors employed in this work conforms whenever possible to the recommendations of the Symposium on Endemiology of Cancer of the Lung [1] and the classification offered by Liebow [49] but modified in respect to cylindromatous carcinoma by the findings of Enterline and Schoenberg.[17] We reluctantly adhere to the term, "bronchial adenoma," because of usage, recognizing, with others,[64] the malignant potential of this tumor.

All of the histologic material on all of the tumor cases was reviewed independently by two of us who are pathologists (F. C. C. and H. T. E.), with special emphasis being placed on a search for blood vessel invasion. We agreed that the criteria for tumor invasion of a blood vessel must include demonstration of either direct growth of tumor through the entire media and intima of a vessel or demonstration of a histologic relationship of tumor within a vessel lumen to the wall of the vessel at some point. Thus, tumor within the media alone was not considered as invading the given vessel, nor were loose tumor cells within the lumina accepted as representing invasion because of possible confusion with artifacts. As expressed by Meissner,[50] the "tumor must be in a vessel and look as if it is at home there." Independent agreement on the presence of absence of blood vessel invasion was reached in 97.4% of the cases.

Results

The incidence of the various tumor types in the 226 resected primary pulmonary neoplasms is seen in Table 1. Over half of the cases available for study were classified as squamous-cell carcinomas, a finding similar to most other reported series. We regarded

TABLE 1.—*Incidence of Tumor Types in 225 Patients with 226 Resected Tumors*

Primary Lung Neoplasm	Total	Male	Female
Squamous-cell carcinoma	116	110	6
Adenocarcinoma *	49 *	32	17
Undifferentiated carcinoma	28	24	4
Mixed carcinoma	12	12	0
Bronchiolar carcinoma	11	7	4
Carcinoid adenoma *	9 *	5	4
Cylindromatous carcinoma	1	1	0

*In the resected specimen from one male there were two tumors, adenocarcinoma and carcinoid adenoma.

as mixed carcinomas only those tumors showing significant combinations of both adenoid and epidermoid elements but with no clear evidence as to unilateral histogenesis. This parallels earlier observations made by Legg.[37] As stressed by McGrath, Gall, and Kessler [46] and others,[73] if enough mucicarmine stain or the Alcian blue [53,54] technique showed evidence of mucin production within individual tumor cells and were classified as adenocarcinomas. An occasional poorly differentiated epidermoid carcinoma could be recognized for what it was only after phosphotungstic acid-hematoxylin stains [41] made clear the intercellular bridges.

Table 3 indicates the incidence of vascular invasion in the different types of tumors, and, as can be seen, vascular invasion more or less parallels the degree of dedifferentiation of the primary pulmonary neoplasm. Thus all of the undifferentiated carcinomas evidenced vascular invasion. The bronchiolar carcinomas, regarded in general as relatively well-differentiated carcinomas, showed evidence of vascular invasion in only slightly over one-half of the cases; however, the

TABLE 2.—*Comparison of Frequency of Different Histologic Types in Several Representative Series*

Author	Year	Total Cases Studied	Tumor Types, %		
			Undifferentiated Carcinoma	Adenocarcinoma	Squamous-Cell Carcinoma
Olson	1935	69	33	24	43
Koletsky	1938	100	38	22	40
Hollingsworth	1947	231	35.1	19.5	42.4
Moritz	1950	100	41	22	37
Dungal	1950	12	25	42	0
Reingold et al.	1950	60	30	3.3	66.7
McDonald et al.	1951	1849	49	13.2	37.8
Doll & Hill	1952	1465	40.5	8.5	38
Banker	1955	43	65	28	7
Collier et al.	1957	218	12.8	20.6	52.8

levels of most adenocarcinomas are studied, areas of squamous metaplasia will be seen. Such cases with only occasional foci of squamous metaplasia were not classified as mixed tumors, a term reserved in our hands for those tumors in which the pattern of growth was fairly evenly divided into squamous and glandular patterns.

The fact that the present series shows a lower percentage of undifferentiated carcinomas than do most other [5,14,15,31,36,44,52,57,62] series (Table 2) is perhaps best interpreted as a reflection of the exacting criteria by which we defined undifferentiated carcinoma. Many poorly differentiated carcinomas, when subjected to the Mayer [43] number of cases of this type, in this series, is small.

Of the first 225 patients with resected primary lung cancers, 100 were operated upon sufficiently remotely to afford opportunity for five-year follow-up. Of these

TABLE 3.—*Incidence of Blood Vessel Invasion by Tumor Type in 225 Patients*

Tumor Type	Incidence	%
Cylindromatous carcinoma	0 of 1	0
Carcinoid adenoma	1 of 9	11
Bronchiolar	6 of 11	54
Squamous-cell carcinoma	74 of 116	64
Adenocarcinoma	42 of 49	86
Mixed carcinoma	11 of 12	92
Undifferentiated	28 of 28	100

TABLE 4.—*Comparison of Five-Year Survival with Blood Vessel Invasion in Ninety-Three Patients*

Tumor Type	Cases, No.	Blood Vessel Invasion	5-Year Survival
Squamous-cell carcinoma	55	36	19
Adenocarcinoma	23	19	2
Undifferentiated carcinoma	7	7	0
Mixed carcinoma	3	2	1
Bronchiolar carcinoma	1	0	0
Carcinoid adenoma	3	1	2
Cylindromatous carcinoma	1	0	1
Total	93	65	25

100 cases, 2 patients died within the first 24 hours, while 5 more died within the first month and were also classified as operative deaths. Although there were differences in five-year survival of patients with varying histologic types (Table 4), in general, the correlation between histologic types and survival was not evident, except that none of the seven patients with undifferentiated carcinoma survived five years.

However, a sharp disparity exists between the survival rate of those patients whose tumors had invaded blood vessels and those patients whose tumors were not associated with blood vessel invasion. As seen in Table 5, the striking difference between the total number of five-year survivors in the group showing vascular invasion and those in the group in which vascular invasion was not demonstrated begins to become apparent at the end of the first year after operation. A second observation which can be made from this Table is that survival at three years is practically identical to survival at five years.[67]

Of a total of 93 patients followed for more than five years after operation, 61 (94%) of 65 patients with vascular invasion were dead within five years, while only 8 (28%) of 28 patients without vascular invasion failed to survive this period.

TABLE 5.—*Excised Primary Lung Tumor—Five-Year Follow-Up in Ninety-Three Patients*

	Cases, No.	Survival, % 1 yr.	3 yr.	5 yr.
Tumors with vascular invasion	65	42	8	6
Tumors without vascular invasion	28	83	72	72

TABLE 6.—*Incidence of Vascular Invasion by Age Groups*

Age	Angio-Invasive Carcinomas, %
40	71.4
41-50	61
51-60	68.5
61-70	72.4
71	83.3

The incidence of blood vessel invasion showed no correlation with the age of the patient (Table 6).

Over half of the total number of cases with blood vessel invasion also had regional lymph node involvement. Conversely, of those patients whose regional nodes were involved by tumor, over three-quarters also showed vascular invasion. It is possible that were more sections available for study, vascular invasion could have been demonstrated in additional cases, and, therefore, accounted for some of the deaths in the patients in whom vascular invasion was not found.

Of the resected patients who subsequently expired, 30 came to autopsy at this hospital or elsewhere. The sections from each of the autopsies were reviewed, and in 19 of the 30 patients metastatic carcinoma was present. In all but two of these patients, vascular invasion had been demonstrated in the surgically excised specimens. Of the 11 patients without autopsy evidence of metastatic disease, 6 showed vascular invasion in the surgical specimen but none survived longer than seven weeks postoperatively. Indeed, five of the six patients were classified as operative deaths. These early deaths defy estimation of the probability of metastasis had survival been longer.

The obviously grave prognosis associated with vascular invasion prompted a further search into the group in which vascular invasion was not observed to see if there were

factors that might account for the demise of 25% of this latter group. Twelve of the twenty-eight cases in whom vascular invasion was not found showed other evidence of extension beyond the gross confines of the primary tumor. Such evidence consisted of the presence of tumor in the line of resection, the demonstration at operation of invasion of intrathoracic structures other than the lung, or invasion of regional lymph node. Subtraction of these 12 cases from the 28 cases in which vascular invasion was not found left 16 patients with tumor, which we have designated as tumors which were purely localized.

It should be stressed that the term "purely localized," in this presentation, is not employed in the usual sense wherein a localized tumor is used as a synonym for a small resectable tumor removed at an operation regarded as a curative procedure. We reserve the term "purely localized" for those tumors which were not found by the surgeon at the time of operation to be associated with invasion of intrathoracic structures other than the lung, where radiologic studies failed to demonstrate the presence of tumor in sites other than the primary lesion, and, most important, in those in which after complete evaluation by the pathologist the lines of resection were free of tumor, regional nodes were not involved, and vascular invasion was not present. Employing these strict criteria, as seen in Table 7, 16 cases fell into this rather strict category. In these 16 cases there was only 1 death within five years, a 94% five-year survival rate.

TABLE 7.—*Survival Following Resection of Tumors Without Angio-Invasion in Twenty-Eight Patients*

Extent of Tumor	Cases, No.	Survival, %		
		1 yr.	3 yr.	5 yr.
Not localized	12	67	50	50
Purely localized	16	100	94	94

Comment

It is a maxim in oncology that in general the smaller and more localized the malignancy, the more amenable it is to effective therapy. We take no issue with this generalization. Thus Gibbon et al.[24] have emphasized pulmonary confinement as the most important single factor mediating favorable prognosis. We would agree that confinement determines prognosis. Moreover, blood vessel invasion is the most important determinant of "confinement," since, in addition to being occult, it constitutes the major pathway for metastasis. Since, as a result of this study, blood vessel invasion has seemed so important in the determination of the prognosis, the burden of proof that no vascular invasion is present is on the pathologist. Thus if the initial blocks do not show vascular invasion, additional sections should be submitted until the pathologist is reasonably certain that further studies would not show blood vessel invasion.

In experimental cancer, as well as in human cancer, no constant relationship can be demonstrated between the size of the primary neoplasm and the number of metastases.[63,81] These observations were confirmed in a corollary manner by this present study, in which no constant correlation could be found between the size of the tumor and the presence or absence of vascular invasion. Sixty per cent of tumors less than 10 cc. showed vascular invasion, and the same incidence was found in those tumors ranging from 200-1500 cc., while 82% of tumors in the range from 11-200 cc. were associated with vascular invasion. It should be emphasized, again, that the property of vascular invasion could not be demonstrated as being dependent upon the so-called central or peripheral location of the individual lung cancer.

Although the present study points to vascular invasion as a most important criterion affecting prognosis in any given case of resected lung cancer, the question of what induces vascular invasion in one tumor and prevents it in another is not answered. It is only possible to state that

the ability which a tumor possesses to invade vascular structures might be regarded as representing increased growth potential, since those tumors invading blood vessels are biologically more malignant than those which do not. The alternate possibility is that a tumor which fails to invade vascular structures fails to do so because of some factor present in the host which increases the host resistance. It cannot be shown that host resistance is dependent on age or that malignant potential is dependent upon size, location, or (except in the undifferentiated carcinomas) histologic type; hence, the stimuli for, or factors influencing, vascular invasion are still undetermined.

Statistical Evaluation

It is patently not possible in a study of this sort adequately to control all variables to the point where there can be absolute predictability of accuracy. The nature of the observations of vascular invasion is such that the errors would always be errors of exclusion rather than of false inclusion. Since adequate control observations cannot be made, the only test for errors of exclusion must rest in autopsy study of patients whose tumors were sampled incident to the project. Therefore, of the 30 autopsy patients whose surgical resected tumors were studied, the 11 showing no residual tumor at time of autopsy are of little benefit to the present test. Of 19 who showed autopsy evidence of tumor, all but 2 showed evidence of vascular invasion by tumor in the surgical specimen. The reasons most apropos for missing vascular invasion in these two specimens are two. First, there may have been failure to select the proper block. Second, even though at autopsy there was evidence of blood vessel invasion, such evidence may not have existed at the time of surgery and tumor may have invaded vascular structures directly from areas of lymphogenous metastasis. Assuming that the second factor did not obtain, we were interested in testing the bias of sample based on the 95% confidence interval of the ratio of negative surgically resected specimens to positive autopsy material.

Obviously, in such a small series as 19 autopsy patients, the confidence interval is great—1 to 31; however, were the same proportion of negative surgical specimens to positive autopsy specimens extended to 1000 positive autopsies, the interval is 9 to 13.

Testing the hypothesis that there were no significant differences in the vascular invasive ability of the different types of primary pulmonary cancers was effected by treating the tumors as samples from a binomial distribution. In lieu of an independent estimate of probability, 0.72 was used as the probability for invasion, as shown in Table 3, where 75% of all the lung tumors studied showed vascular invasion (Table 8).

In Table 5 the survival of patients whose tumors invaded blood vessels is contrasted with the survival of those whose tumors were not associated with vascular invasion. Statistical evaluation, effected by treating the tumors as samples from a binomial distribution, was predicated on rejecting the hypothesis that there were no differences in survival between the invasive and noninvasive tumors (Table 9).

TABLE 8.—*Probability of Blood Vessel Invasion* *

Tumor type	Cases with Blood Vessel Invasion (r), No.	Total Cases Studied (n)	Probability of at Most r
Cylindromatous carcinoma	0	1	0.280
Carcinoid adenoma	1	9	0.000
Bronchiolar carcinoma	6	11	0.168
Epidermoid carcinoma	74	116 (using 120)	0.056
Adenocarcinoma	42	49	0.993
Mixed carcinoma	11	12	0.981
Undifferentiated carcinoma	28	28	1.000

* Small probabilities, as shown here, indicate either that there is a significant difference in vascular invasive ability of the different tumor types or that a rare event in sampling has occurred.

TABLE 9.—*Differences in Survival Between Invasive and Noninvasive Tumors* *

Blood Vessel Invasion	Total Cases (n), No.	Survivors (r), No.			Probability of at Most r		
		1 yr.	3 yr.	5 yr.	1 yr.	3 yr.	5 yr.
+	65	27	5	4	0.022	0.000	0.000
—	28	23	20	20	1.0	1.0	1.0

* In lieu of an independent estimate of probability, the probability for the computation was based on the percentage of survivors of all cases sampled at the stated time intervals. Small probabilities, as shown in the blood vessel invasion + group, indicate that the difference in survival between the + and the — groups are of statistical significance.

Estimation of the practical application of this study to future studies of individual patients with resected lung cancer is important in the light of the statistical significance of the correlation of prognosis with vascular invasion. Such estimation is predicated on the validity of sampling and the adequacy of sampling. In the present work, since the number of samples necessary was unknown, we adhered to Warren's statement regarding sampling for blood vessel invasion in thyroid cancers, in which he cited work that three blocks were adequate.

In the present data, the only controls available for analysis for adequacy of sampling are in the autopsy series, in which vascular invasion was found in 23 surgical specimens. In two additional cases there is autopsy evidence of metastasis by vascular invasion. Assuming that the vascular invasion was missed because of inadequacy of sampling, the percentage of false negative observations was 8% in this small series. Extending this observation to cover the 225 patients studied herein, the 95% confidence interval is 5 to 13. Therefore, assuming the maximum in false negatives compounded by the maximum in the confidence interval, a minimum of 15% of lung cancers will not show blood vessel invasion, when the maximum of 13% is added to the 72% of all resected lung cancers in which blood vessel invasion was observed.

Summary

The clinical and pathological data on 226 consecutive resected lung carcinomas were studied. A 100% follow-up was obtained.

The most important factor influencing prognosis was found to be the presence or absence of blood vessel invasion. Only 6% of patients whose tumors had invaded blood vessels were alive at the end of five years, while 72% of patients whose tumors were not associated with vascular invasion survived five or more years.

By rigidly limiting the term "undifferentiated carcinoma" to those malignant epithelial tumors in which differentiation in any known direction cannot be shown and by utilizing histochemical techniques to reveal occult evidence of differentiation, we considerably reduced the percentage of undifferentiated tumors in this series. No patient with an undifferentiated carcinoma so defined survived for five years. All of the

TABLE 10.—*Statistical Significance in Survival Between Patients with Not Localized and Those with Localized Cancers*

Extent of Tumors	Total Cases (n), No.	Survivors (r), No.			Probability of at Most r		
		1 yr.	3 yr.	5 yr.	1 yr.	3 yr.	5 yr.
Not localized	12	8	6	6	0.075	0.054	0.054
Purely localized	16	16	15	15	1.000	0.999	0.999

* This Table, set up in much the same fashion as Table 9, with the data analyzed by the same technique, may be interpreted as showing the statistical significance in the survival between the two groups of patients — those with not localized and those with purely localized cancer.

tumors in this group were associated with vascular invasion—usually of striking degree.

Tumors can be considered as localized only if there is no evidence of extension of tumor beyond the confines of the primary site. The factors excluding a tumor as localized include peri- or parapulmonary invasion, tumor in the lines of resection, and tumor in regional lymph nodes. The most frequently missed excluding factor is vascular invasion. The importance of accurate definition and strict adherence of the term "localized tumor" is manifest in the 94% five-year survival of patients with a tumor so designated.

Statistical evaluations of the data and conclusions are presented.

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Lydia G. Kinzer, Statistician, Statistics Laboratory, Department of Mathematics, The Ohio State University, did the statistical studies.

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News and Comment

ANNOUNCEMENTS

Training Fellowships in Chemical Pathology.—Fellowships for training in chemical pathology are being offered by the Tulane Department of Pathology in cooperation with the Department of Biochemistry. The fellowships are designed to prepare selected medical graduates for careers in academic pathology and research. In a period of four years after internship, a fellow should be able to fulfill the requirements for board certification in pathologic anatomy and, if desired, a doctorate in biochemistry. Candidates with previous experience in pathology may be admitted to advanced standing. The program for each fellow will be adapted to his individual needs, interests, and previous training but will include experience in postmortem and surgical pathology, graduate work in biochemistry, and research experience in experimental pathology. Special emphasis will be placed on integrating gross and histopathologic changes with abnormalities disclosed by electron microscopy, cellular biochemistry, and histochemistry. Candidates for fellowships will be expected to have a solid background in college chemistry, a better than average record in medical school, and an interest in remaining in academic work. Fellowship stipends will begin at $4200. Financial support for this program has been provided by the Rockefeller Foundation and the National Cancer Institute.

Applications or requests for further information should be addressed to Charles E. Dunlap, M.D., Department of Pathology, Tulane University School of Medicine, 1430 Tulane Ave., New Orleans 12.

SOCIETY NEWS

American Academy of Oral Pathology.—At the annual meeting of the American Academy of Oral Pathology, in Washington, D. C., new officers were elected and installed. They are as follows: President, Dr. Charles Waldron; President-Elect, Col. Joseph Bernier, U. S. Army; Vice-President, Capt. Robert Colby, U. S. Navy; Secretary-Treasurer, Dr. Robert J. Gorlin.

Application for membership and fellowship are made through the office of the secretary-treasurer. Address all inquiries to Dr. Robert J. Gorlin, Secretary-Treasurer, American Academy of Oral Pathology, School of Dentistry, University of Minnesota, Minneapolis 14.

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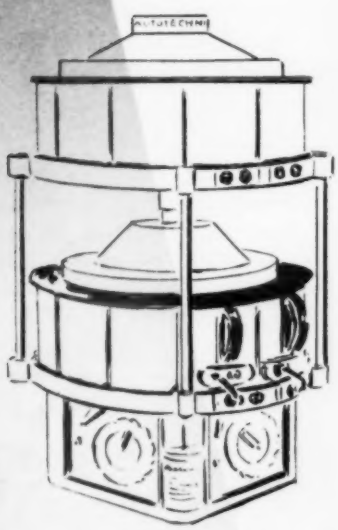

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